

High Pathogenic Potential of Low-Affinity Autoantibodies in Experimental Autoimmune Hemolytic Anemia

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Summary

To assess the potency of low-affinity anti-red blood cell (RBC) autoantibodies in the induction of anemia, we generated an immunoglobulin (Ig)G2a class-switch variant of a 4C8 IgM anti-mouse RBC autoantibody, and compared its pathogenic potential with that of its IgM isotype and a high-affinity 34-3C IgG2a autoantibody. The RBC-binding activity of the 4C8 IgG2a variant was barely detectable, at least 1,000 times lower than that of its IgM isotype, having a high-binding avidity, and that of the 34-3C IgG2a monoclonal antibody (mAb). This low-affinity feature of the 4C8 mAb was consistent with the lack of detection of opsonized RBCs in the circulating blood from the 4C8 IgG2a-injected mice. However, the 4C8 IgG2a variant was highly pathogenic, as potent as its IgM isotype and the 34-3C IgG2a mAb, due to its capacity to interact with Fc receptors involved in erythrophagocytosis. In addition, our results indicated that the pentameric form of the low-affinity IgM isotype, by promoting the binding and agglutination of RBCs, is critical for its pathogenic activity. Demonstration of the remarkably high pathogenic potency of low-affinity autoantibodies, if combined with appropriate heavy chain effector functions, highlights the critical role of the Ig heavy chain constant regions, but the relatively minor role of autoantigen-binding affinities, in autoimmune hemolytic anemia.

Key words: autoantibody • autoimmune hemolytic anemia • Fc receptor • Kupffer cell • knockout mouse

The pathogenesis of autoantibody-mediated cellular and tissue lesions in autoimmune diseases is most straightforwardly attributable to the combined action of self-antigen binding properties and effector functions associated with the Fc regions of the different Ig isotypes, such as the capacity to bind Fc receptors for IgG (Fc γ R),¹ activate complement, and cause multivalence-induced agglutination. This is best exemplified by remarkable differences among a panel of Coombs' anti-RBC monoclonal autoantibodies, established from lupus-prone NZB mice, in their ability to induce autoimmune hemolytic anemia (1–5).

It has been believed that the high-affinity binding of autoantibodies to self-antigens is likely to be critical for the expression of their pathogenic activities in vivo. This notion has been supported by the demonstration that the Ig genes in a subset of autoantibody-producing B cells, which are clonally expanded, are modified by somatic mutations, es-

pecially in their CDR3 (6, 7), and that the affinity maturation of autoantibodies in association with IgM to IgG class switching coincides with progression of the clinical development of autoimmune diseases, most notably SLE (8, 9). However, the affinity maturation of autoantibodies in parallel to Ig class switch and somatic hypermutations is not a consistent observation. Analysis of clonally related anti-IgG2a rheumatoid factors isolated from a lupus-prone MRL-*lpr/lpr* mouse has demonstrated only limited affinity maturation, despite Ig isotype switching with extensive somatic mutations (10). Most significantly, studies on a panel of anti-IgG2a rheumatoid factor monoclonal autoantibodies disclosed that even low-affinity autoantibodies are able to induce immune complex-mediated vasculitis, with a cryoglobulin activity uniquely associated with murine IgG3 isotype (11, 12).

Recent studies on anti-mouse RBC monoclonal autoantibodies revealed a major role of Fc γ R-mediated erythrophagocytosis in the development of anemia induced by IgG anti-RBC autoantibodies (13, 14). Thus, it can be speculated that low-affinity anti-RBC autoantibodies of IgG isotypes could become highly pathogenic, if combined with the

¹Abbreviations used in this paper: Fc γ R, Fc receptor for IgG; FcR γ , FcR γ chain; HE, hematoxylin and eosin; Ht, hematocrit(s); NHS-biotin, N-hydroxysuccinimide biotin.

capacity to interact with Fc γ R expressed on phagocytic effector cells. To explore this possibility, we have generated an IgG2a class-switch variant from an NZB-derived 4C8 IgM anti-mouse RBC monoclonal autoantibody (2). Since it has been generally thought that the IgM antibody has a low affinity, we should be able to study the pathogenic activity of low-affinity anti-RBC autoantibody of IgG2a isotype capable of interacting with phagocytic Fc γ R (13, 14). In the present work, comparative analysis of the 4C8 IgG2a variant with its IgM isotype and a high-affinity 34-3C IgG2a anti-mouse RBC monoclonal autoantibody, established from NZB mice (4), demonstrates that despite a low binding affinity, both 4C8 IgM and IgG2a isotypes are remarkably pathogenic, because of the high-avidity binding capacity of polyvalent IgM isotype or because of a high-affinity interaction of IgG2a isotype with Fc γ R involved in erythrophagocytosis.

Materials and Methods

Mice. BALB/c mice were purchased from Bomholtgard. Mice deficient in FcR γ chains (FcR γ), which lack functional expression of both Fc γ R type I (Fc γ RI) and type III (Fc γ RIII), and wild-type littermates were developed as described previously (15).

DNA Construction. The VDJH4C8-C γ 2a plasmid containing the complete 4C8 IgG heavy chain gene of the IgG2a isotype was constructed using the following DNA fragments: the rearranged VDJ region isolated from cDNA encoding the variable region of the heavy chain of the 4C8 mAb (16), the promoter region isolated from pSV-V μ 1 (17), the heavy chain enhancer region isolated from pSVE2-neo (18), and the C γ 2a region derived from the genomic clone, pIgH10 (19).

mAbs. Hybridomas secreting the 4C8 IgM and 34-3C IgG2a anti-mouse RBC mAbs were derived from unmanipulated NZB mice (2, 4). The 4C8 IgG2a class-switch variant was obtained by transfecting 4C8 heavy chain loss mutant cells by electroporation with the VDJH4C8-C γ 2a plasmid together with a pSVE2-neo plasmid containing the neomycin-resistant gene. After selection for resistance to neomycin and secretion of IgG antibodies, stable transfected cells secreting the 4C8 IgG2a variant were cloned by limiting dilutions. IgG2a anti-TNP (Hy1.2) and IgM anti-IgG2a (6-19) mAbs were used as control. Rat anti-mouse κ chain mAb (H139.52.1.5) was provided by Dr. M. Pierres (Centre d'Immunologie de Marseille-Luminy, Marseille, France [20]). IgG mAbs were purified from culture supernatants by protein A column chromatography. IgM mAbs were purified by euglobulin precipitation from culture supernatants concentrated by 50% saturated ammonium sulfate precipitation, according to the method of Garcia-Gonzales et al. (21). The purity of IgG and IgM was >90% as documented by SDS-PAGE.

Reverse Transcriptase PCR and cDNA Sequencing. RNA was prepared from 4C8 IgG2a-transfected cells by RNeasy Mini Kit (Qiagen). The first strand of cDNA was synthesized with an oligo(dT) primer and 5 μ g of total RNA. For amplification with *Pfu* DNA polymerase (Stratagene), the following primers were used: 5'-untranslated VH primer (5'-CAGTTCTCTACAGTTA-3') and C γ 2a-CH1 primer (5'-GCCAGTGGATAGAC-3') for the 4C8 heavy chain; 5'-untranslated V κ primer (5'-CAGGGGAAGCAAGATGG-3') and C κ primer (5'-TGGATGGTGGAAGATG-3') for the 4C8 light chain. The nucleotide se-

quence corresponding to the variable region of the 4C8 heavy or light chain was determined by the dideoxynucleotide chain terminating method (22).

Anti-Mouse RBC Assays. A flow cytometric assay was used to detect anti-mouse RBC activities of purified mAbs. 50 μ l of various concentrations of purified mAbs was incubated with 20 μ l of 1% RBC suspension, prepared from BALB/c mice, in 1% BSA-PBS for 30 min at 4°C. After washing three times with 1% BSA-PBS, the RBCs were incubated with biotinylated rat anti-mouse κ chain mAb (H139.52.1.5), followed by PE-conjugated streptavidin R (Caltag), and analyzed with a FACScan™ (Becton Dickinson). The presence of opsonized RBCs in mice injected with anti-mouse RBC mAb was detected by a similar flow cytometric assay, using biotinylated rat anti-mouse κ chain mAb. For in vitro and in vivo bindings of anti-mouse RBC mAb on biotinylated mouse RBCs, bound antibodies were revealed with PE-labeled F(ab')₂ goat anti-mouse Ig conjugates (Jackson Immuno-Research Labs). An indirect radioimmunoassay was also used to detect anti-mouse RBC activities of mAbs, as described previously (4). In brief, 100 μ l of various concentrations of purified mAbs was incubated with 10 μ l of 25% RBC suspension in 1% BSA-PBS for 30 min at 4°C. After washing three times with 1% BSA-PBS, the RBCs were incubated with ¹²⁵I-labeled goat anti-mouse Ig in 1% BSA-PBS. Results are expressed as the radioactivity (in cpm) bound to RBCs. Complement-mediated lysis and hemagglutination assays were as described previously (4).

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. Blood samples were collected into heparinized microhematocrit tubes and centrifuged in a hematocrit centrifuge, and hematocrits (Ht) were directly determined after centrifugation, as described previously (4).

In Vivo Biotinylation of Mouse RBCs. Mouse RBCs were biotinylated in vivo by an intravenous infusion of *N*-hydroxysuccinimide biotin (NHS-biotin; Boehringer Mannheim), according to the method described by Dale and Daniels (23). In brief, 2 mg NHS-biotin was dissolved in 0.12 ml *N,N*-dimethylacetamide (Sigma Chemical Co.), incubated for 2 min in the dark at 37°C, and added to 3.2 ml sterile saline. 500 μ g of NHS-biotin was then injected intravenously into mice. This in vivo biotinylation procedure resulted in essentially all the RBCs being biotinylated, as judged by staining with streptavidin Red 670 (GIBCO BRL) 24 h after the infusion of NHS-biotin.

Histological Studies. Livers and spleens were obtained at autopsy, processed for histological examination, and stained with hematoxylin and eosin (HE). The extent of in vivo RBC destruction by Kupffer cell-mediated phagocytosis was revealed by coloration of liver sections with Perls iron staining.

Results

Low-Affinity Binding to Mouse RBCs by the 4C8 IgG2a Isotype-Switch Variant. The strategy for generating the recombinant IgG2a isotype-switch variant was to clone the heavy chain variable region (VH) gene from the 4C8 IgM anti-mouse RBC hybridoma and to join this gene to the already cloned mouse IgG2a heavy chain constant region gene. The recombinant IgG2a isotype-switch variant was then expressed in a heavy chain loss mutant of the 4C8 IgM hybridoma. Notably, the VH4C8 and V κ 4C8 sequences of the 4C8 heavy and light chain cDNA derived from a reverse transcription PCR amplification of mRNA

isolated from the cells secreting 4C8 IgG2a switch variant were identical to the originally published sequence (16).

The *in vitro* binding activity of the 4C8 IgG2a switch variant, compared with its IgM isotype and the high-affinity 34-3C IgG2a anti-mouse RBC mAb, was investigated by flow cytometric analysis using a biotinylated rat anti-mouse κ chain mAb, followed by PE-conjugated streptavidin. Incubation of mouse RBCs with 0.1 ng of the 4C8 IgM or 34-3C IgG2a mAb resulted in substantial binding, whereas >250 ng of the IgG2a switch variant was required to show significant binding, with a maximal binding at 1 μ g (Fig. 1). Thus, mouse RBC-binding activity of the 4C8 IgG2a variant was at least 1,000 times less than that of its IgM isotype and the 34-3C IgG2a mAb. It should also be mentioned that the staining intensity obtained by the 4C8 IgG2a variant was far lower than that observed with the 4C8 IgM or the 34-3C IgG2a mAb. Marginal RBC-binding activities by the 4C8 IgG2a variant were confirmed with a radioimmunoassay (Fig. 2). These results indicated that the 4C8 mAb has a low binding affinity, while its binding activity was markedly promoted by the pentameric high-avidity IgM isotype. Moreover, hemagglutination activity was only detectable with the 4C8 IgM mAb, and neither IgM nor IgG2a 4C8 mAb exhibited complement-mediated hemolysis *in vitro* in the presence of guinea pig, rabbit, or mouse serum (data not shown).

Lack of Detection of Opsonized RBCs, but Efficient Elimination of Circulating RBCs in Mice Injected with the Low-Affinity 4C8 IgG2a Variant. Because of its markedly limited *in vitro* binding to mouse RBCs, we determined whether the 4C8 IgG2a variant was capable of binding to circulating RBCs *in vivo*. When analyzed by a flow cytometric assay 24, 48, and 72 h after a single intraperitoneal injection of 1 mg of the 4C8 IgG2a variant into BALB/c mice, opsonized RBCs were undetectable in the circulating blood (Fig. 3 A). This was in marked contrast to the presence of bound antibodies on essentially all the circulating mouse RBCs between 24 and 72 h after the administration of 50 μ g of

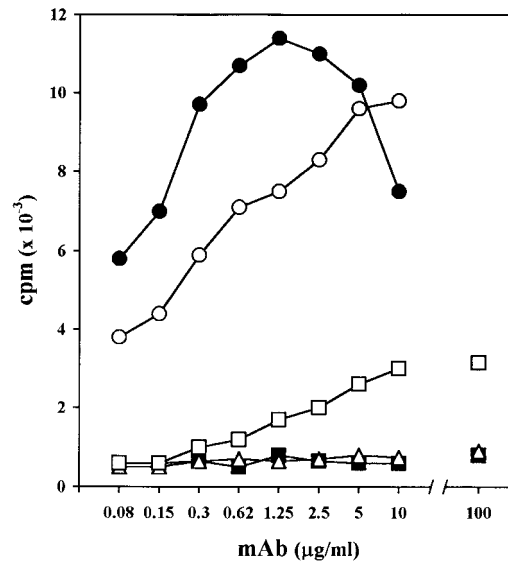


Figure 2. Radioimmunoassay for the detection of anti-mouse RBC activities by the 4C8 (IgM and IgG2a) and 34-3C (IgG2a) anti-mouse RBC mAbs. RBCs freshly prepared from BALB/c mice were first incubated with different concentrations of various mAbs, and then incubated with radiolabeled goat anti-mouse Ig. The results are expressed as the radioactivity bound to mouse RBCs in the presence of 4C8 IgM (●), 4C8 IgG2a (□), and 34-3C IgG2a (○). As controls, the results obtained with isotype-matched control mAbs are shown: IgM anti-IgG2a (■) and IgG2a anti-TNP (△).

the 4C8 IgM or 34-3C IgG2a mAb. Notably, the staining pattern obtained by *in vitro* incubation with the 4C8 IgG2a variant was indistinguishable between RBCs from mice injected with the 4C8 IgG2a and those from control mice, excluding a rapid and selective elimination of a subpopulation of circulating RBCs by the low-affinity 4C8 IgG2a (data not shown). Thus, the lack of detection of opsonized RBCs in the circulating blood further confirmed a low-affinity feature of the 4C8 IgG2a switch variant.

Strikingly, mice injected with 1 mg of the 4C8 IgG2a

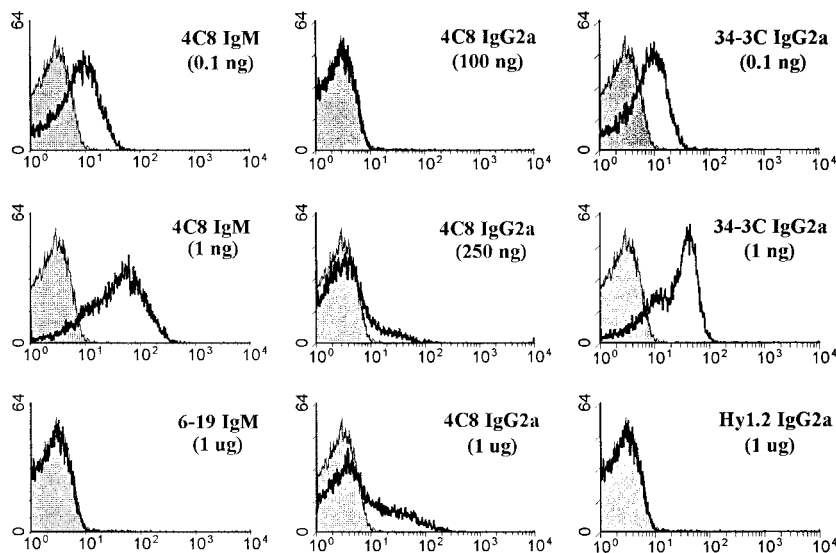


Figure 1. Flow cytometric analysis of mouse RBCs stained with different amounts of the 4C8 (IgM and IgG2a) and 34-3C (IgG2a) anti-mouse RBC mAbs. RBCs freshly prepared from BALB/c mice were first incubated with different amounts of various mAbs, and then incubated with biotinylated anti-mouse κ chain mAb, followed by PE-conjugated streptavidin. Shaded histograms indicate the background staining with anti-mouse κ chain mAb alone. As controls, the results obtained with isotype-matched control mAbs are shown.

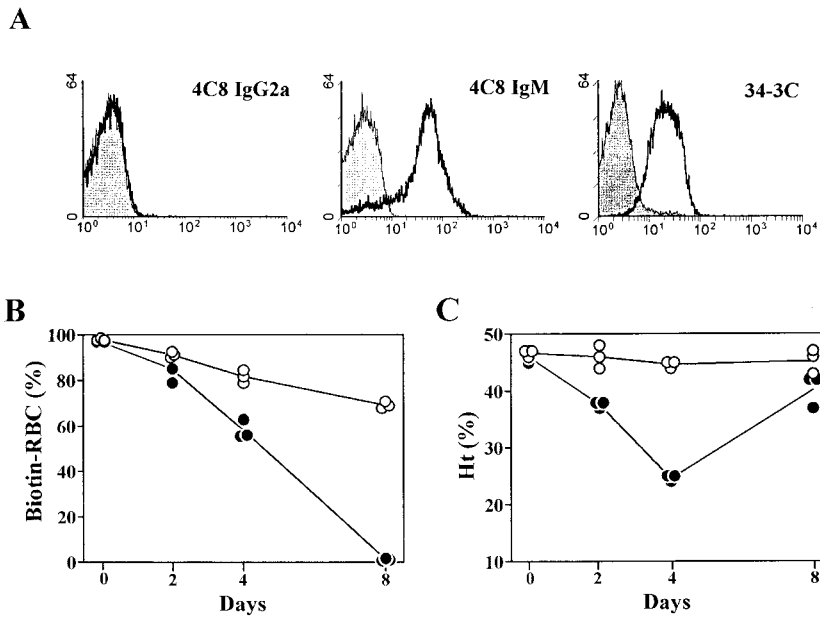


Figure 3. Failure to detect opsonized circulating RBCs, but development of anemia accompanied by efficient clearance of circulating RBCs after injection of the 4C8 IgG2a variant in BALB/c mice. (A) Mouse RBCs were obtained 24 h after an intraperitoneal injection of 4C8 IgG2a (1 mg), 4C8 IgM (50 μ g), or 34-3C IgG2a (50 μ g) anti-mouse RBC mAb, and then stained with biotinylated anti-mouse κ chain mAb, followed by PE-conjugated streptavidin. Shaded histograms indicate the background staining with PE-conjugated streptavidin. Essentially identical results were obtained with mouse RBCs obtained 48 and 72 h after injection of anti-mouse RBC mAb (data not shown). (B and C) Mouse RBCs were biotinylated in vivo by an intravenous infusion of NHS-biotin 24 h before the intraperitoneal injection of 1 mg of purified 4C8 IgG2a (●) or control IgG2a Hy1.2 anti-TNP mAb (○) on day 0. The clearance of biotinylated RBCs was followed by the staining with streptavidin Red 670. Results are expressed as percent biotinylated RBCs (Biotin-RBC) of three individual mice, measured 2, 4, and 8 d after the mAb injection (B). The development of anemia was determined by measuring Ht values (C).

variant developed a severe anemia with a decrease in Ht values down to 21% 4 d after the injection (see below). To verify the elimination of circulating RBCs by the 4C8 IgG2a variant, RBCs were in vivo biotinylated with an intravenous injection of NHS-biotin, and their clearance was then determined after the injection of 1 mg of 4C8 IgG2a or Hy1.2 IgG2a anti-TNP mAb. As shown in Fig. 3 B, biotinylated RBCs were gradually replaced by newly generated RBCs in control mice injected with Hy1.2 anti-TNP mAb, while the injection of the 4C8 IgG2a variant markedly accelerated this process. Although Ht values began to recover ~5 d after the 4C8 IgG2a mAb injection and returned to almost normal levels by 8 d (Fig. 3 C), the levels of biotinylated RBCs were further decreased. 8 d after 4C8 IgG2a mAb injection, biotinylated RBCs were hardly detectable (<1%), while ~70% of circulating RBC were still biotinylated in control mice. This indicated that the 4C8 IgG2a variant indeed bound to circulating RBCs in vivo and efficiently eliminated them. Notably, the immunoreactivity of biotinylated RBCs, obtained from 24 to 72 h after in vivo biotinylation, to the 4C8 mAb was comparable to that of control RBCs, as judged by in vitro and in vivo binding analyses, and the kinetics and extent of anemia induced by the 4C8 mAb in biotin-treated mice were essen-

tially identical to those of untreated mice (Fig. 3 C and Fig. 4).

High Pathogenic Activity of the Low-Affinity 4C8 IgG2a Variant Promoted through Fc γ R-dependent Erythrophagocytosis.

To compare the pathogenic activity of the 4C8 IgG2a variant with its IgM isotype and the high-affinity 34-3C IgG2a mAb, the development of anemia was analyzed by a single intraperitoneal injection of various amounts of purified mAb into BALB/c mice. Despite low-affinity binding to mouse RBCs, the 4C8 IgG2a mAb was highly pathogenic, and 50 μ g was sufficient to cause significant anemia (mean Ht values of five mice 4 d after the injection: $35 \pm 3\%$; Fig. 4). This dose was comparable to that required for the induction of anemia by its IgM isotype and the high-affinity 34-3C IgG2a mAb.

Histological examinations showed that erythrophagocytosis by Kupffer cells was the most remarkable pathological change associated with anemia induced by the 4C8 IgG2a isotype (Fig. 5 A), as observed in mice developing anemia after the injection of the 34-3C IgG2a mAb (4). Destruction of RBCs by phagocytosis was further documented by extensive iron deposits in Kupffer cells from mice injected with the 4C8 IgG2a variant (Fig. 5 C). In contrast, the injection of 4C8 IgM mAb at a dose of 250 μ g resulted in an enormous accumulation of agglutinated RBCs in the spleen,

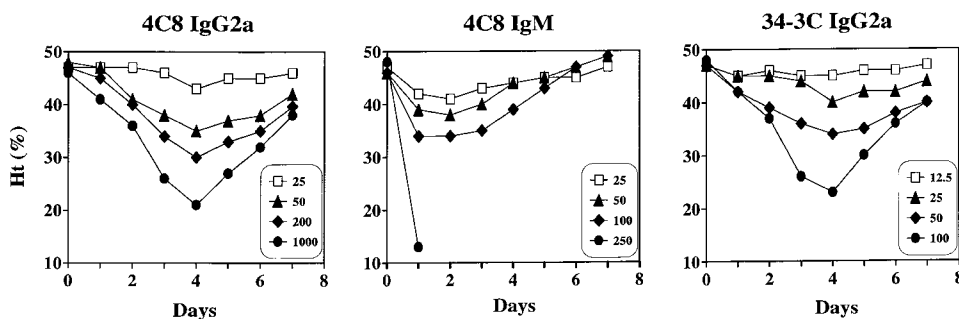


Figure 4. Development of anemia by the 4C8 and 34-3C anti-mouse RBC mAbs in BALB/c mice. Mice were intraperitoneally injected with different amounts of purified mAbs on day 0: 25 μ g, 50 μ g, 200 μ g, or 1 mg of 4C8 IgG2a mAb; 25, 50, 100, or 250 μ g of 4C8 IgM mAb; 12.5, 25, 50, or 100 μ g of 34-3C IgG2a mAb. Results are expressed as mean Ht values of three to five mice.

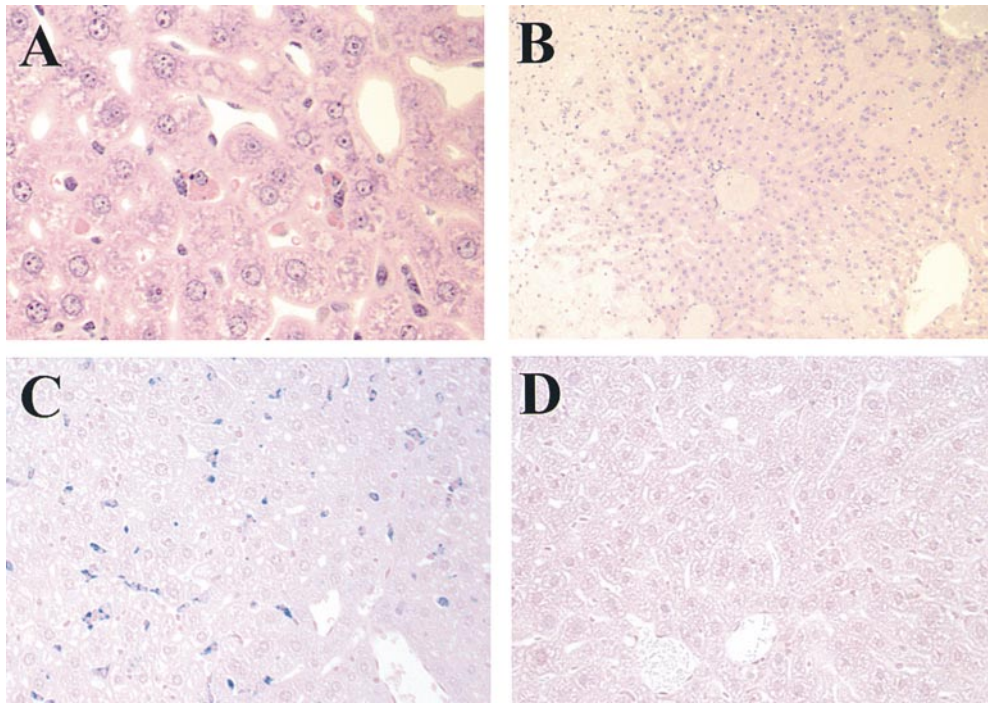


Figure 5. (A) Representative histological appearance of liver 4 d after the injection of 1 mg of the 4C8 IgG2a variant. Note the presence of marked erythrophagocytosis by Kupffer cells (HE; original magnification: $\times 400$). (B) Representative histological appearance of liver from BALB/c mice that died of anemia 2 d after the injection of 250 μg of the 4C8 IgM mAb. Note an enormous accumulation of agglutinated RBCs in sinusoids of liver accompanied by necrosis of hepatic parenchymal cells (HE; original magnification: $\times 100$). (C and D) Representative histological appearance of iron deposits in Kupffer cells from BALB/c and FcR γ -deficient mice after the injection of 1 mg of the 4C8 IgG2a variant. Mice injected with the 4C8 IgG2a variant were killed at day 7. The extent of in vivo RBC destruction by phagocytosis was revealed by coloration of liver sections with Perls iron staining. Note extensive iron deposits in Kupffer cells from BALB/c mice injected with the 4C8 IgG2a mAb (C), in marked contrast to the complete absence of iron deposits in livers from FcR γ -deficient mice injected with the 4C8 IgG2a mAb (D) (original magnification: $\times 200$).

and in hepatic sinusoids accompanied by occasional necrosis of hepatic parenchymal cells (Fig. 5 B), as shown previously (4).

To determine the contribution of Fc γ R-mediated erythrophagocytosis to the pathogenesis of the 4C8 IgG2a-induced anemia, the development of anemia was assessed in FcR γ -deficient mice lacking functional expression of both Fc γ RI and Fc γ RIII involved in phagocytosis of IgG2a-opsonized RBCs (13, 14). As shown in Fig. 6, FcR γ -deficient mice were completely resistant to the pathogenic effect of 1 mg of the 4C8 IgG2a mAb, and failed to exhibit erythrophagocytosis, as documented by the lack of iron deposits in their Kupffer cells (Fig. 5 D).

Discussion

In this study, we have demonstrated that the 4C8 anti-mouse RBC autoantibody, derived from lupus-prone NZB mice, has a low binding affinity to its corresponding self-antigen, yet its IgM and IgG2a isotypes exhibit a remarkably high pathogenic potency. Our results indicate that the pentameric form of a low-affinity IgM isotype, by promoting the binding and agglutination of RBCs, is critical for its pathogenic activity, and that the capacity of the IgG2a isotype to interact with Fc γ R involved in erythrophagocytosis is responsible, despite its barely detectable RBC-binding activity, for its hemolytic activity. Demonstration of the high

pathogenic potency of low-affinity autoantibodies highlights a remarkable role of Ig heavy chain effector functions, as opposed to a relatively minor role of autoantigen-binding affinities, in the pathogenesis of autoimmune hemolytic anemia.

A striking feature of our analysis is that despite the lack of detection of significantly opsonized RBCs in the circu-

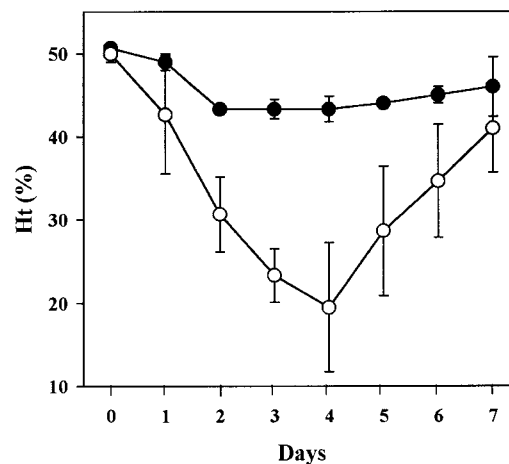


Figure 6. Development of anemia in FcR γ -deficient (●) and wild-type (○) mice after the injection of 1 mg of the 4C8 IgG2a variant. Mean Ht values (± 1 SD) of three mice are shown.

lating blood, the 4C8 IgG2a variant with low binding affinity is able to induce a severe anemia as a result of peripheral destruction of circulating RBCs. This was documented by an efficient clearance of *in vivo*-biotinylated circulating RBCs and by Kupffer cell-mediated erythrophagocytosis. To our surprise, the amount of the IgG2a isotype-switch variant required to induce anemia was comparable to that of the high-avidity IgM isotype exhibiting >1,000 times stronger RBC-binding activity. This indicates that the pathogenic activity of low-affinity IgG anti-RBC autoantibodies is dramatically enhanced when provided with an appropriate Ig heavy chain effector function, namely the capacity to interact with Fc γ R involved in erythrophagocytosis. Thus, a rapid Fc γ R-mediated uptake by Kupffer cells of even very poorly opsonized RBCs present in hepatic microcirculation appears to be an extremely efficient mechanism for the development of anemia induced by IgG anti-RBC autoantibodies.

An additional, unexpected observation was that the pathogenic potential of the low-affinity 4C8 IgG2a autoantibody was also comparable to the high-affinity 34-3C IgG2a mAb (4). However, this comparison is only tentative in its interpretation because of possible differences in the specificity of the 4C8 and 34-3C mAbs (24). Only experiments comparing IgG2a mAbs of the same specificity, but with different affinities, would provide definitive conclusions on this issue. Nevertheless, our results strongly suggest that RBC-binding affinities of Coombs' autoantibodies apparently play a relatively minor role for *in vivo* hemolytic activities of the IgG2a isotype capable of interacting efficiently with two different classes of Fc γ R (Fc γ RI and Fc γ RIII) involved in erythrophagocytosis (13, 14, 25, 26). It may be worth noting that although the pathogenicity of the 4C8 and 34-3C IgG2a mAbs was almost comparable at lower doses, it appears that the 34-3C mAb at higher doses is more pathogenic than the 4C8 IgG2a (Fig. 4). Although we do not have a straightforward explanation for this phenomenon at present, it can be speculated that the IgG2a isotype of anti-RBC autoantibodies could mediate erythrophagocytosis through their binding to the high-affinity Fc γ RI, followed by their subsequent interaction with circulating RBCs. A significant binding of free IgG2a anti-RBC to Fc γ RI on phagocytic cells is likely to occur only when higher doses of IgG2a mAb are injected, because of the competition of circulating monomeric IgG2a without anti-RBC activity. Owing to its high-affinity RBC-binding capacity, even limited amounts of the cell-bound 34-3C IgG2a may efficiently capture circulating RBCs, thereby mediating erythrophagocytosis. In contrast, the ability to capture circulating RBCs by the low-affinity 4C8 IgG2a bound on Kupffer cells may be too poor to cause erythrophagocytosis by such a mechanism. Consistent with this hypothesis, our recent analysis on different Fc γ R-deficient mice has shown that the erythrophagocytosis caused by lower doses of the 34-3C and 4C8 IgG2a mAbs is primarily mediated by the low-affinity Fc γ RIII, while Fc γ RI is only involved in the development of anemia induced by higher doses of these mAbs (our unpublished results).

In addition to the remarkable contribution of IgG Fc region mediating Fc γ R interaction to pathogenic activities of the low-affinity 4C8 IgG2a variant, these studies also suggest that the strong pathogenic activity of the IgM 4C8 isotype is dependent on its pentameric form, which promotes the binding and agglutination of RBCs. Consequently, the IgM isotype of the 4C8 mAb induces a different form of severe anemia, resulting from massive agglutination of RBCs in spleen and liver, which does not involve Fc γ R-mediated phagocytosis and complement activation (4, 13). Although the present study does not formally prove that the pentameric form of the 4C8 IgM antibody is crucial for the *in vivo* agglutination of RBCs, we have recently observed that the IgG3 variant of the 4C8 mAb exhibits neither Fc γ R-dependent erythrophagocytosis nor agglutination of RBCs *in vivo* (our unpublished results), supporting the importance of the IgM pentameric form for the *in vivo* agglutination of RBCs by this mAb. However, it should be stressed that we have previously observed that two of four IgM anti-RBC mAbs failed to induce anemia (4). The lack of pathogenic activities by these two IgM autoantibodies is likely to be related to their specificities, since they are unable to induce agglutination of mouse RBCs *in vitro*. Thus, the surface density of corresponding self-antigen epitopes may be too low to promote high-avidity binding and agglutination of mouse RBCs *in vivo* by these IgM anti-RBC autoantibodies.

Our demonstration here of the high pathogenic potency of low-affinity autoantibodies suggests that the affinity maturation of autoantibodies may not be a critical process for the generation of autoantibodies with immunopathologic consequences. This is consistent with the demonstration that low-affinity anti-IgG2a rheumatoid factors of the IgG3 isotype are able to induce remarkable pathology—immune complex-mediated skin vasculitis and lupus-like glomerulonephritis—owing to their cryoglobulin activity, a unique property of the IgG3 isotype (11, 12). These results provide new insight into the cellular basis for the generation of pathogenic autoantibodies of IgM and IgG isotypes. It has already been shown that a fraction of B cells expressing the low-affinity 4C8 autoantibody can escape clonal deletion in the bone marrow and can be activated to produce pathogenic autoantibodies in the periphery, as a result of nonspecific activation of B cells (27, 28). These autoantibodies may even be switched to IgG classes under certain conditions, possibly through the action of the cytokines, independently of the presence of autoantigen-specific T helper cells, as is the case of T cell-independent type II immune responses (29). Genetic abnormalities present in certain autoimmune-prone mice may favor the switching of IgM to IgG isotypes, as shown by a spontaneous class switch from IgM to IgG2a autoantibody by B cells derived from lupus-prone (NZB \times NZW)F1 mice in the absence of functional CD4⁺ T helper cells (30, 31). In addition, we have recently observed that the constitutive expression of the *bd-2* transgene in germinal center B cells is able to induce the spontaneous production of IgG autoantibodies (our unpublished results). Therefore, it is possible that autoreactive B cells can be generated

as a result of somatic hypermutations in the germinal centers during immune responses against environmental antigens, and such B cells may persist, if they are defective in the process of apoptosis, which is likely to be one of the genetic defects present in autoimmune-prone mice (32, 33). Thus, although the presence of somatic mutations in Ig variable regions, including CDR, of autoantibodies is likely to be CD4⁺ T cell dependent, several autoantibody-producing B cells may not have been driven by autoreactive T helper cells and self-antigens. This idea is consistent with a limited

affinity maturation of clonally related rheumatoid factors, despite extensive somatic mutations, derived from a Fas-deficient MRL-*lpr/lpr* mouse (10). Clearly, in view of the high pathogenic potential of low-affinity autoantibodies and of the critical role of CD4⁺ T cells in the development of autoimmune diseases (34), better understanding of the nature of help provided by CD4⁺ T cells would help elucidate the cellular basis central to the development of autoantibody-mediated autoimmune diseases.

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