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A Novel Antibody against Human Factor B that Blocks Formation of the C3bB Proconvertase and Inhibits Complement Activation in Disease Models

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The alternative pathway (AP) is critical for the efficient activation of complement regardless of the trigger. It is also a major player in pathogenesis, as illustrated by the long list of diseases in which AP activation contributes to pathology. Its relevance to human disease is further emphasized by the high prevalence of pathogenic inherited defects and acquired autoantibodies disrupting components and regulators of the AP C3-convertase. Because pharmacological downmodulation of the AP emerges as a broad-spectrum treatment alternative, there is a powerful interest in developing new molecules to block formation and/or activity of the AP C3-convertase. In this paper, we describe the generation of a novel mAb targeting human factor B (FB). mAb FB48.4.2, recognizing with high affinity an evolutionary-conserved epitope in the Ba fragment of FB, very efficiently inhibited formation of the AP C3-proconvertase by blocking the interaction between FB and C3b. In vitro assays using rabbit and sheep erythrocytes demonstrated that FB28.4.2 was a potent AP inhibitor that blocked complement-mediated hemolysis in several species. Using ex vivo models of disease we demonstrated that FB28.4.2 protected paroxysmal nocturnal hemoglobinuria erythrocytes from complement-mediated hemolysis and inhibited both C3 fragment and C5b-9 deposition on ADP-activated HMEC-1 cells, an experimental model for atypical hemolytic uremic syndrome. Moreover, i.v. injection of FB28.4.2 in rats blocked complement activation in rat serum and prevented the passive induction of experimental autoimmune Myasthenia gravis. As a whole, these data demonstrate the potential value of FB28.4.2 for the treatment of disorders associated with AP complement dysregulation in man and animal models. *The Journal of Immunology*, 2014, 193: 000–000.

Complement is an essential component of innate immunity and a major trigger of inflammatory responses. It plays a crucial role in microbial killing, apoptotic cell clearance, immune complex handling and modulation of adaptive immune responses (1, 2). Complement is initiated by three activation pathways, the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP). The critical step in these activation pathways is the formation of labile protease complexes, termed C3-convertases (C3bBb in the AP; C4b2a in the CP/LP) that cleave C3 to generate the active fragment, C3b. When C3b is generated, a reactive thioester is exposed, which permits covalent binding of C3b to the activating surface, targeting it for destruction and initiating inflammation. The incorporation of one additional C3b

molecule to the AP C3-convertase creates the C5-convertase, which cleaves C5 thereby triggering inflammation and leukocyte recruitment through production of C5a. Cleavage of C5 also initiates formation of the membrane attack complex (MAC) (3).

The efficiency of complement activation relies on the AP amplification loop in which the C3b generated by the C3-convertase forms more AP C3-convertase and provides exponential amplification to the initial activation. The balance between the rate at which the initial trigger is amplified and the degree to which C3b and the C3-convertases are inactivated determines the progression of the complement cascade to cell damage and death. Foreign sugars on microbial pathogens (AP), Abs (CP), or mannan (LP) tip the balance in favor of amplification, causing target opsonization

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M.S., M.D., and S.R.d.C. conceived and planned the project; M.S., A.T., S.G., M.G., A.L.-P., L.d.J.L., F.A.G.-F., M.D., O.L., A.V.-M., M.N., B.P.M., and S.R.d.C. designed and/or performed the research experiments and analyzed the data; and M.S., O.L., M.N., B.P.M., and S.R.d.C. drafted and edited the manuscript.

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Abbreviations used in this article: AChR, acetylcholine receptor; aHUS, atypical hemolytic uremic syndrome; AP, alternative pathway; CP, classical pathway; EAMG, experimental autoimmune MG; FB, factor B; LP, lectin pathway; MAC, membrane attack complex; MG, myasthenia gravis; NHS, normal human serum; PNH, paroxysmal nocturnal hemoglobinuria; RU, resonance unit; SPR, surface plasmon resonance.

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and cell lysis. In health, complement activation is strictly regulated and limited to the activator surface (4).

Pharmacological downmodulation of the AP is predicted to be beneficial in the treatment of a long list of diseases in which complement activation contributes to pathology by sustaining inflammation and perpetuating tissue damage (5). Indeed, inhibition of the AP is emerging as the treatment of choice in diseases, like paroxysmal nocturnal hemoglobinuria (PNH), C3 glomerulopathies, atypical hemolytic uremic syndrome (aHUS), and Myasthenia gravis (MG), in which dysregulation of the AP C3 convertase, because of inherited defects or acquired autoantibodies, is the etiopathogenic factor (6–9).

Previously, mAbs specific for either FB or C3b that block formation of the AP C3-convertase (C3bBb) have been generated and provided proof of concept in models that inhibition of the AP prevents or ameliorates diseases like antiphospholipid Ab syndrome, complement-mediated hemolytic anemia or ischemia/reperfusion injury (10–16). In this paper, we report the generation of a novel mAb against human factor B (FB) with capacity to block the activity of the AP. FB48.4.2, a mAb that recognizes with high affinity an evolutionarily conserved epitope in the Ba fragment of human FB, inhibits complement activation by blocking the formation of the AP C3 proconvertase.

Materials and Methods

Isolation of complement components

C3 was prepared by an established protocol involving ammonium sulfate precipitation, anion exchange chromatography (DEAE and Mono S; GE Healthcare), and size exclusion chromatography (Superdex 200 Increase 10/300 GL; GE Healthcare) (17); FB was affinity purified from EDTA plasma on anti-Bb (mAb D2, in-house), followed by anion exchange chromatography (Mono Q; GE Healthcare) and size exclusion chromatography (Superdex 200 Increase 10/300 GL; GE Healthcare). FD was purchased from Calbiochem. C3b was generated by coincubating pure C3, FB, and FD as previously described (17), followed by anion exchange chromatography (Mono Q; GE Healthcare) and size exclusion chromatography (Superdex 200 Increase 10/300; GE Healthcare). Concentrations of pure proteins were assessed using absorbance at $A_{280\text{nm}}$ and the following extinction coefficients: FB: $1.43\text{cm}^{-1}(\text{mg/ml})^{-1}$, C3: $0.98\text{cm}^{-1}(\text{mg/ml})^{-1}$, and Ig $1.4\text{cm}^{-1}(\text{mg/ml})^{-1}$.

Generation of mAbs

FB-deficient mice (a gift from Prof. M. Botto, Imperial College, London UK) were immunized with 20 μg human FB emulsified with CFA and then boosted three times at 2-wk intervals with the same amount of FB with incomplete adjuvant. The mice were screened for the development of Abs to FB by testing their sera in an ELISA using human FB-coated plates. Positive mice were given an additional boost with 20 μg of a mixture of Ba and Bb fragments in PBS. Three days later, spleen cells from a mouse having a robust immune response toward FB were fused to the x63AG8 myeloma cell line. Candidate hybridomas were cloned by limiting dilution, and clones recognizing human FB by ELISA were identified and expanded. Abs were purified from tissue culture supernatants with a protein G-Sepharose column (Pharmacia, Uppsala, Sweden). The purity of the mAbs was then analyzed by 10% SDS-PAGE, and their Ig isotype was determined with the IsoStrip Mouse mAb Isotyping Kit (Roche Applied Science). The mAb products of all selected hybridomas were tested in ELISA and/or Western blot for their capacity to detect FB in human, mouse, rat, rabbit, and pig sera. All animal experimentation has been reviewed and approved by the Consejo Superior de Investigaciones Científicas Institutional Review Board.

FB and C3 cleavage assays

The effects of mAbs on FB and C3 cleavage were tested in an in vitro assay using purified C3, FB and FD because all preparations of C3 contain sufficient C3(H₂O) to trigger formation of the AP convertase. Briefly, C3 (0.55 μg), FB (0.5 μg), and FD (0.001 μg) were mixed in 25 μl AP buffer (2.9 mM barbital, 1.7 mM sodium barbital, 144 mM NaCl, 7 mM MgCl₂, and 10 mM EGTA [pH 7.4]). Increasing amounts of each mAb anti FB (from 0.5 to 2 μg) were added to the reaction mix, which was then incubated at 37°C for 30 min in a water bath. Cleavage of FB and C3 was assessed after 10% SDS-PAGE under reducing conditions and Coomassie blue staining.

Hemolysis assays

The capacity of the mAbs to inhibit the AP on cellular surfaces was assessed in a hemolytic assay using rabbit erythrocytes. Titration of normal human serum (NHS) for lysis of rabbit erythrocytes was performed before the experiment to determine the optimal conditions for the hemolysis inhibition assay. A final concentration of 2.5% of NHS was used in our experiments, as this amount was just sufficient to completely lyse the rabbit erythrocytes under the conditions used. Experiments were done in triplicate with each reaction containing 100 μl rabbit erythrocytes ($1 \times 10^8/\text{ml}$) in isotonic GVB buffer (AP buffer containing 0.1% gelatin), 100 μl 5% NHS in AP buffer and either eculizumab (Soliris, Alexion Pharmaceuticals) or FB28.4.2 at 37°C for 30 min at 37°C with occasional shaking. The reactions were stopped by adding 2 ml ice-cold saline. Tubes were centrifuged at $1000 \times g$ for 5 min at 4°C, and the supernatants read at 414 nm. Erythrocytes with NHS and 20 mM EDTA were used as a blank and erythrocytes with NHS and water as 100% lysis.

Rabbit erythrocytes were also used to test the capacity of the mAbs to prevent C3 deposition on cell surfaces. Briefly, 100 μl rabbit erythrocytes ($1 \times 10^8/\text{ml}$) in AP buffer were incubated with 5% NHS in presence of either eculizumab (Soliris, Alexion Pharmaceuticals) or FB28.4.2 at 37°C for 30 min. C3 deposition was evaluated by flow cytometry using a rabbit polyclonal anti human C3 Ab (in house; 1 $\mu\text{g}/\text{ml}$ in PBS) or the mouse anti-human iC3b/C3dg mAb SIM320.12.2.1 (in house; 0.5 $\mu\text{g}/\text{ml}$ in PBS).

The capacity of the mAb to inhibit the AP on cell surfaces was also assessed in a FH-dependent hemolytic assay using sheep erythrocytes and a well-characterized serum from an aHUS patient carrying the FH-W1183L mutation (18). In brief, $1 \times 10^8/\text{ml}$ sheep erythrocytes were incubated with 20% patient serum in AP buffer with increasing concentrations of FB28.4.2 or control mAb for 30 min at 37°C. Percent lysis was calculated as described above.

The capacity of the mAb FB28.4.2 to inhibit acidified lysis of PNH erythrocytes (the Ham test) (19) was assessed by incubating (37°C for 1 h) PNH erythrocytes (4% v/v final) with 25% NHS in AP buffer acidified by addition of 9% HCl 0.2 M in the presence of increasing amounts of FB28.4.2 (from 9 $\mu\text{g}/\text{ml}$ [0.06 μM] to 75 $\mu\text{g}/\text{ml}$ [0.5 μM]). Hemolysis in supernatants was read at 540 nm, and percent lysis was calculated using 0 and 100% lysis controls.

The capacity of FB28.4.2 to inhibit hemolysis in sera from other species was tested in hemolytic assays essentially as described above. For rat and pig sera, rabbit erythrocytes in AP buffer were used and the serum doses selected (just sufficient to cause 100% lysis) were 7.5% (rat) and 10% (pig). For mouse serum, Ab-sensitized rabbit erythrocytes in CP buffer were used as described (20) with a selected serum dose of 4%. Dose of mAb was varied as described.

Surface plasmon resonance studies

All analyses were carried out on a Biacore x100 (GE Healthcare). For the kinetic characterization of human FB binding to mAb FB28.4.2, we used a single-cycle-kinetics method. This approach consisted of five consecutive injections of FB (analyte) at increasing concentrations over a surface in which the FB28.4.2 Ab was captured using an anti-mouse Ab. Each injection lasted for 150 s separated by a dissociation period of 180 s, during which FB-free buffer was injected. The cycle was completed with an extended dissociation period of 1300 s and a regeneration step to release both the FB28.4.2 and FB. This protocol is essentially based on the same principles as a traditional multicycle analysis (in which after concentration injection and dissociation period, there is a regeneration step) and can be described with the same equations but using a modified model supported by the Biacore software. The model is based on a one-to-one binding interaction, each analyte injection is individually fitted, and the total response was calculated. The numerical model considers the amount of analyte bound to Ab in the previous injection and its dissociation and also contains terms for mass transport, drift, and bulk refractive index mismatches. Importantly, this, multicycle approach provides kinetic rate constants that are very similar to those obtained using the single-cycle approach (21, 22).

For the functional analysis of the mAb FB28.4.2, C3b (1000 resonance units [RU]) was amine-coupled to a CM5 (carboxymethylated dextran) sensor chip as instructed by the manufacturer (NHS/EDC coupling kit; GE Healthcare). To characterize the binding of FB to C3b in the absence or presence of FB28.4.2, FB was flowed across the chip for 120 s alone or premixed with up to a 4-fold molar excess of mAb; binding was allowed to decay for 300 s. The chip surface was regenerated by flowing 20 mM EDTA. To characterize the binding kinetics of FB to the mAbs, 300 RU mAb was coupled to a CM5 sensor chip using the Mouse Ab capture Kit (BR-1008-38; GE Healthcare); increasing concentrations of FB were flowed in the presence of 20 mM HEPES, 150 mM NaCl, and 5 mM MgCl₂ for 150 s and allowed to decay for 1300 s. The chip surface was regenerated with 50 mM

sodium acetate (pH 4.2) and 50 mM NaOH. All data were double-referenced (data from control cell and blank injection subtracted). Data were evaluated using Biaevaluation software (version 4.1; GE Healthcare).

Flow cytometry assays

EDTA blood was collected from eculizumab-treated PNH patients or healthy individuals after informed consent. Erythrocytes were harvested by centrifugation, washed with PBS several times until the supernatant remained clear, and stored for up to 1 wk in ACD-A buffer at 4°C. C3 fragments deposition on the PNH erythrocytes membrane was measured by flow cytometry. Briefly, a 0.4% suspension of erythrocytes was incubated with a rabbit polyclonal anti human C3 Ab (in-house; 1 µg/ml) or the mouse anti-human iC3b/C3dg mAb clone SIM320.12.2.1 (in-house; 0.5 µg/ml in PBS) for 30 min at room temperature and then with an anti-rabbit or an anti-mouse IgG Ab labeled with Alexa 488 (Life Technologies) 0.5 µg/ml in PBS.

C3 fragment and C5b-9 deposition on HMEC-1 cells

The human microvascular endothelial cell line of dermal origin HMEC-1 was cultured as described (23). HMEC-1 were plated on glass slides and used when confluent. Cells were activated with 10 µM ADP (Sigma-Aldrich) for 10 min, thereafter cells were incubated for 4 h with serum from an aHUS patient in the presence or absence of the FB28.4.2 mAb or in the presence or the absence of sCR1 (150 µg/ml; Celldex). At the end of the incubation step HMEC-1 were fixed in 3% paraformaldehyde and stained with FITC-conjugated rabbit anti-human C3c-complement (DakoCytomation) or with rabbit anti-human C5b-9 (Calbiochem) followed by FITC-conjugated secondary Ab (Jackson ImmunoResearch Laboratories). A confocal inverted laser microscope (LSM 510 Meta; Zeiss) was used for acquisition of the fluorescent staining on endothelial cell surface. Fifteen fields, systematically digitized along the surface, were acquired using a computer-based image analysis system. The area occupied by the fluorescent staining was evaluated by automatic edge detection using built-in specific functions of the software ImageJ (National Institutes of Health, Bethesda, MD), and expressed as pixel² per field analyzed. For each sample the mean of 15 fields (excluding the lowest and the highest values) was calculated. Results are expressed as mean ± SE. Data were analyzed by ANOVA. A *p* value < 0.05 was considered to be statistically significant.

Testing FB28.4.2 in experimental autoimmune MG

Experimental autoimmune MG (EAMG) was passively induced in young adult female Lewis rats essentially as described (20, 24). In brief, rats were weighed and prebled, then injected i.p. with the antiacetylcholine receptor (AChR) mAb35 (1 mg/kg in PBS). At the same time as disease induction, rats were injected i.v. with FB28.4.2 mAb (8 mg in 1 ml PBS; six rats) or PBS as control (1 ml PBS; six rats). Rats were monitored for signs of clinical disease and weighed at 4, 24, and 48 h postdisease induction and bled at 4 h and at the time of sacrifice. Rats were sacrificed when disease score exceeded 3 (total hind limb paralysis) and/or weight loss exceeded 20% of starting weight; all remaining rats were sacrificed at 48 h. Soleus muscles were harvested from all rats at sacrifice and flash frozen in OCT medium for histological analyses. Serum obtained preinduction, at 4 h, and at sacrifice was tested for hemolytic activity as described above. Muscle was sectioned (10 µm) and stained with rhodamine-labeled bungarotoxin to identify AChR-positive muscle endplates. Intact endplates were counted in five randomly selected fields at ×20 magnification from each muscle section.

Results

Generation and characterization of a mouse mAb targeting human FB that inhibits activation of the complement alternative pathway

Mouse mAbs to human FB were generated as described in *Materials and Methods*. Reasoning that mAbs directed to evolutionarily conserved epitopes were likely to be targeting functional domains in FB, we immunized FB-deficient mice and mAbs from resulting hybridomas were tested for their capacity to recognize both human and mouse FB in ELISA and Western blot. Intact FB and both Ba and Bb fragments were included in these analyses. In addition, all mAb were tested for their capacity to block FB cleavage by FD in the presence of C3b. FB28.4.2 (IgG2b) was

selected for further characterization because it recognized an epitope in the Ba fragment of FB (Fig. 1A) with very high affinity (K_D 3.55 nM; Fig. 1B) and cross-reacted with FB in primate, mouse (weakly), rat, rabbit, goat, sheep, and pig serum, indicating that it detected an evolutionarily conserved epitope (Fig. 1C); furthermore, this mAb blocked the FD-mediated cleavage of FB, preventing the generation of the C3bB convertase (Fig. 1D). The ability of mAb FB28.4.2 to inhibit the AP activity in human, rat, and pig serum was confirmed in hemolysis assays using sheep and rabbit erythrocytes. FB28.4.2 and its Fab fragment inhibited the lysis of rabbit erythrocytes in normal human, rat, and pig serum (Fig. 2A, 2C). Furthermore, FB28.4.2 prevents lysis of sheep erythrocytes in 20% EGTA serum from an aHUS patient carrying the factor H C-terminal mutation W1183L (Fig. 2B). In human serum, 50% inhibition of lysis in these assays was achieved at approximately equimolar concentrations of mAb FB28.4.2 (6 µg/ml, 0.04 µM) and FB (4.5 µg/ml, 0.05 µM) (Fig. 2). Efficiency of inhibition in rat and pig sera was of a similar magnitude, but no inhibition of lysis by mouse serum was seen in the concentration range tested (Fig. 2C).

FB28.4.2 inhibits AP activation by preventing assembly of the C3bB proconvertase

The assays described above indicated that the FB28.4.2 efficiently prevented complement activation by blocking the formation of the AP C3 convertase. To analyze the mechanism by which the FB28.4.2 achieves this inhibition, we performed surface plasmon resonance (SPR) experiments using a C3b-coated amine-coupled CM5 sensor chip. Flowing FB over the sensor chip resulted in binding of FB to C3b and formation of the C3bB proconvertase, which, as expected, was sensitive to accelerated decay by EDTA. Importantly, preincubation of FB with Fab fragment of FB28.4.2, which alone showed no binding to the C3b-coated SPR chip, completely blocked the interaction between C3b and FB (Fig. 3). Previous functional data and structural models have demonstrated that the interaction between C3b and FB involves two interactions: 1) between the von Willebrand type A domain of FB and the C345C domain of C3b and 2) between the Ba domain of FB and the MG2, MG6, MG7, ANA, and CUB domains of C3b (25–29). These data have also shown that the interaction between the Ba domain and C3b is critical for stabilizing an open conformation of FB in the C3bB complex essential for cleavage and activation of FB by FD. In fact, small variations in the K_D values for the interaction between the Ba domain and C3b, for example, those caused by the R32Q polymorphism in the Ba domain of FB, have important consequences for the formation rate of the C3bB proconvertase and activity of the AP (17). In light of these functional and structural data, our SPR results indicate that FB28.4.2 or its Fab fragment binds the Ba domain of FB, blocking formation of the C3bB proconvertase and activation of FB.

FB28.4.2 prevents lysis of PNH erythrocytes in acidified human serum

PNH is a rare complement-mediated hemolytic anemia that arises from an acquired somatic mutation in the *PIGA* gene resulting in the nonmalignant proliferation of hematopoietic stem cell clones and the generation of erythrocytes lacking all GPI-anchored proteins, including the complement regulators CD55 and CD59, which are consequently susceptible to complement-mediated lysis (14). A classical assay to reveal the susceptibility of PNH erythrocytes to complement-mediated lysis, the Ham test (19), involves exposing the PNH erythrocytes to NHS acidified to pH 6.4 to initiate activation of the AP. We have performed the Ham test with erythrocytes obtained from six PNH patients under treatment with

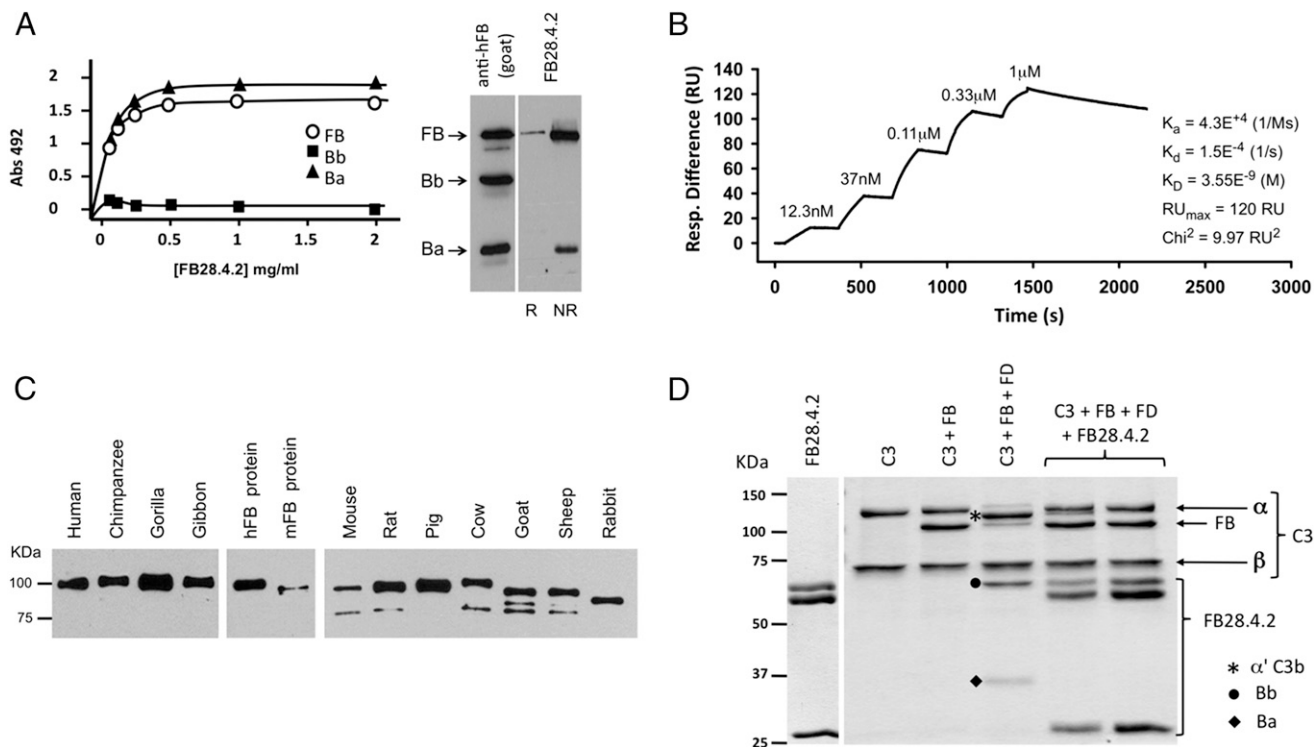


FIGURE 1. FB28.4.2 binds an epitope in the Ba domain of FB and prevents activation of both FB and C3. **(A)** ELISA and Western blot analyses using intact FB and Bb and Ba fragments. Proteins were mixed together prior to SDS PAGE and Western blot. R, reduced conditions. NR, non-reduced conditions. A goat anti-hFB polyclonal Ab was used as a positive control recognizing both Bb and Ba fragments. **(B)** Sensogram showing the kinetic characterization of human FB binding to mAb FB28.4.2 captured with an anti-mouse IgG Ab surface. **(C)** Coomassie-stained gel showing that addition of mAb FB28.4.2 to a mixture of C3 (0.55 μg), FB (0.5 μg), and FD (0.001 μg) in AP buffer prevents cleavage of both FB and C3. **(D)** Western blot analyses of EDTA-plasma samples (0.1 μl) from different mammals to illustrate recognition by FB28.4.2. Purified hFB (0.08 μg) and mFB (0.08 μg) were included for comparison.

eculizumab (Soliris; Alexion Pharmaceuticals), a humanized anti-C5 mAb that inhibits MAC formation by blocking C5 activation. Because these patients are treated with eculizumab, PNH erythrocytes are not lysed and circulate in the plasma of these patients coated with C3 activated fragments; their relative abundance depends on the clone size in each particular patient. C3 deposits on PNH erythrocytes lacking CD59 and CD55 can be demonstrated either by a Coombs direct test or by flow cytometry (Fig. 4, inset). Different percentages of erythrocytes from eculizumab-treated patients, which roughly correlate with the proportion of C3-coated erythrocytes, are lysed when exposed to acidified NHS. Addition of FB28.4.2 inhibited very efficiently the lysis of PNH erythrocytes in all patient samples in a concentration-dependent fashion (Fig. 4). Importantly, 50% inhibition of lysis was achieved in all cases at a concentration of Ab that was essentially equimolar with FB in the serum.

Treatment with eculizumab ameliorates the symptoms associated with chronic intravascular hemolysis and significantly improves the quality of life of PNH patients (30, 31). However, because eculizumab does not block the activity of the AP C3 convertase, C3 opsonization of PNH erythrocytes persists and may result in significant extracellular hemolysis, explaining the persistent hemolytic anemia observed in about half of the PNH patients treated with eculizumab (32, 33). Blocking formation of the AP C3 convertase would improve treatment of PNH by preventing both extravascular and intravascular hemolysis through the inhibition of C3 deposition and MAC formation, respectively.

To investigate the efficacy of FB28.4.2 in preventing opsonization of erythrocytes, we incubated rabbit erythrocytes with NHS in the presence of either eculizumab or FB28.4.2. Flow

cytometry analysis revealed that 100% of rabbit erythrocytes incubated with NHS in the presence of eculizumab were heavily opsonized with C3 fragments; in contrast, no deposition of C3 fragments was detected on erythrocytes exposed to NHS in the presence of inhibitory concentrations of FB28.4.2 (Fig. 5). These data illustrate the potential usefulness of a humanized version of FB28.4.2 in the treatment of PNH patients to prevent both opsonization of the erythrocytes and their complement-mediated lysis.

FB28.4.2 blocks C3 and C5b-9 deposition on activated HMEC-1 cells exposed to serum from aHUS patients

aHUS is a rare, life-threatening disease characterized by thrombocytopenia, hemolytic anemia, and acute renal failure. aHUS is triggered by vascular endothelial damage, which in most cases is associated with mutations and polymorphisms in complement genes. Overwhelming experimental evidence shows that these pathogenic genetic variations confer defective protection of cellular surfaces from complement activation (34). ADP-activated HMEC-1 cells are a good ex vivo aHUS model; they overexpress P-selectin, which works as a receptor for C3b capable of initiating complement activation (35). When these activated cells are exposed to serum from aHUS patients carrying mutations in soluble complement components that impair complement regulation, the surface of these cells becomes covered by C3 fragments and C5b-9 deposits; NHS has no such effect (36, 37). We have used this experimental setting to evaluate the capacity of the FB28.4.2 Ab to prevent complement-mediated endothelial cell damage. When activated HMEC-1 cells were incubated with serum from an aHUS patient carrying a pathogenic *CFH* mutation in the presence of the mAb, both C3 fragment and C5b-9 deposits

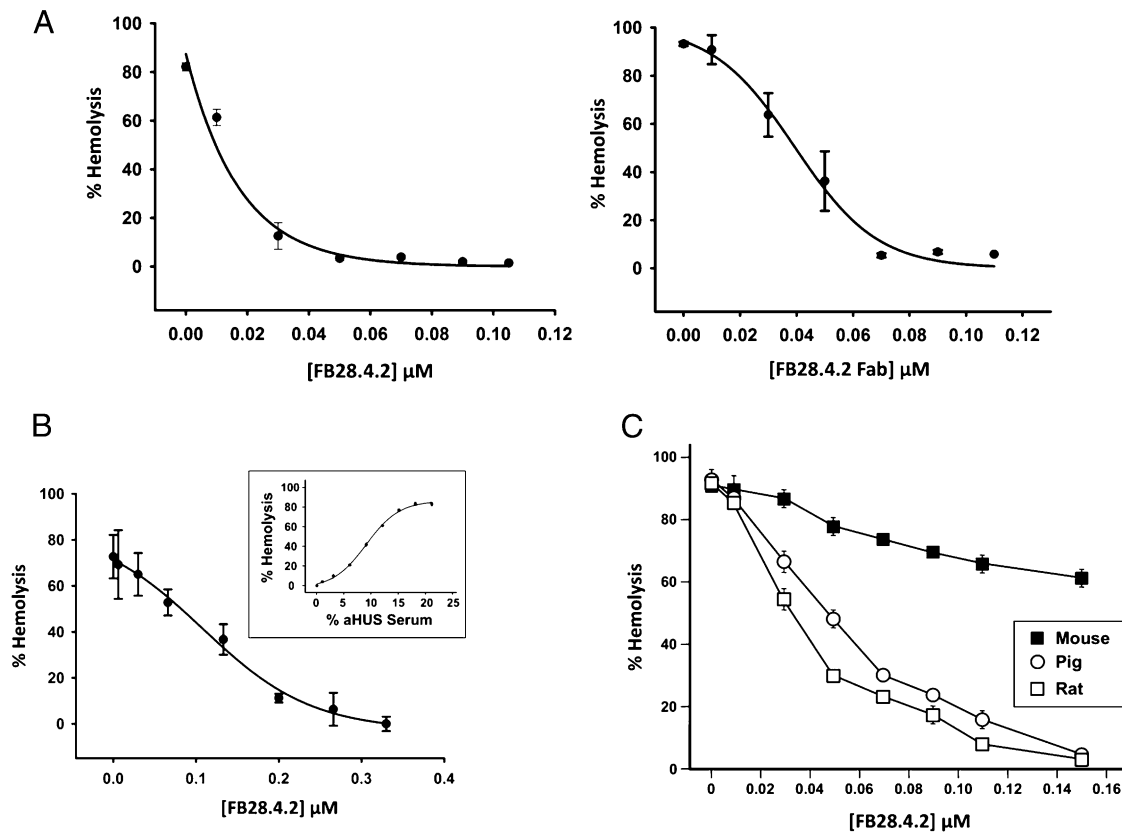


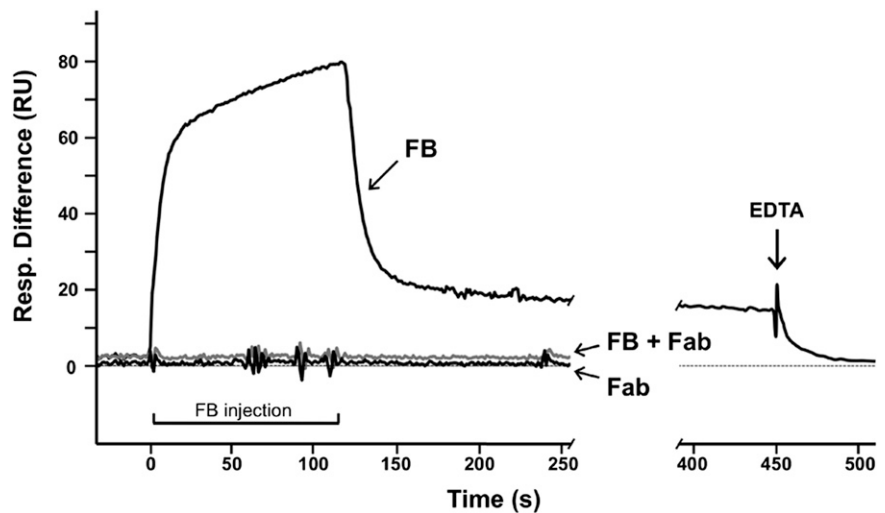
FIGURE 2. FB28.4.2 blocks AP activation and prevents complement-mediate lysis of rabbit and sheep erythrocytes. **(A)** Rabbit erythrocytes activate the AP and are lysed in the presence of human serum. Both FB28.4.2 (*left panel*) and its Fab fragment (*right panel*) inhibited lysis of rabbit erythrocytes in 2.5% of normal human serum. The results represent the mean \pm SD of three independent experiments. **(B)** In contrast to rabbit erythrocytes, lysis of sheep erythrocytes by human serum is factor H dependent. Figure shows that FB28.4.2 prevents lysis of sheep erythrocytes in 20% EGTA serum from an aHUS patient carrying the factor H C-terminal mutation W1183L. The percentage of aHUS required to obtain >80% lysis was determined by exposing sheep erythrocytes to increasing amounts of aHUS serum (*inset*). The results represent the mean \pm SD of three independent experiments. **(C)** Rat (7.5% for 100% lysis) and pig (10%) sera efficiently lysed rabbit erythrocytes under AP conditions; mouse serum (4%) required classical pathway triggering using Ab-sensitized rabbit erythrocytes. FB28.4.2 efficiently inhibited lysis by rat and pig sera but not mouse serum under these conditions. The results represent the mean \pm SD of three independent experiments.

were significantly decreased (Fig. 6). For comparison, we included in these experiments a recombinant truncated form of the membrane-associated complement receptor 1 (sCR1) capable of inhibiting AP activation and decreasing complement deposition on the cell surface. As illustrated in Fig. 6, the results obtained with FB28.4.2 were comparable to those obtained with sCR1.

FB28.4.2 prevents the development of EAMG in rats

MG is an autoimmune disease caused by Abs against the nicotinic AChR. These Abs activate complement at the neuromuscular endplate causing microlysis and reducing the number of AChR, thus hampering the correct transmission of impulses between motor neurons and muscle leading to weakness and fatigability (38–40).

FIGURE 3. FB28.4.2 prevents binding of FB to C3b. Sensogram showing binding of FB (flowed at 0.5 μM) to a CM5 Biacore chip coated with 1000 RU C3b. Preincubation of FB with 4-fold excess of purified FB28.4.2 Fab fragment completely block binding of FB to the chip. Bar indicates the duration of FB injection and the arrow the point of EDTA injection. This experiment was performed twice with identical results.



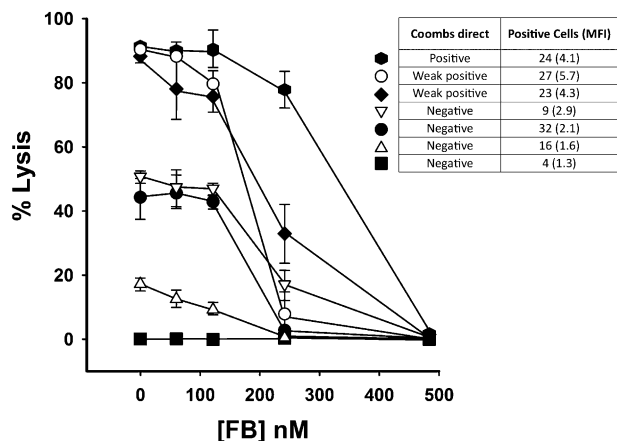


FIGURE 4. FB28.4.2 prevents lysis of PNH erythrocytes in acidified serum. Erythrocytes (8%) from six PNH patients with different sizes of PNH clones and densities of C3 deposits were incubated in 50% acidified NHS alone or in the presence of increasing concentrations of FB28.4.2 mAb. Despite the differences in the percentage of original lysis, which roughly correlates with the size of the PNH clone evident from C3 deposition (demonstrated either by a Coombs direct test (46) or by flow cytometry), FB28.4.2 efficiently prevented the hemolysis in all cases. Data points represent mean \pm SD of three different experiments.

EAMG can be induced actively by AChR immunization or passively by transfer of anti-AChR Abs in mice and rats (20, 24). Strong experimental evidence supports the critical role of complement in the development of both the human disease and the rodent models (7, 41).

We used the passive EAMG model in rats to test whether the FB28.4.2 Ab, inhibitory for rat complement in vitro (Fig. 2C), is able to block the AP in vivo in rats and prevent the development of the disease. EAMG was induced by passive administration of the anti-AChR mAb35. By 24 h after disease induction, all control animals ($n = 6$) receiving the triggering mAb presented substantial weight loss (average, $\sim 10\%$ body mass) (Fig. 7A) and varying degrees of paralysis (Fig. 7B). At this time point, two animals had reached a clinical score of 3 (total hind limb paralysis) and were sacrificed; the remaining four rats progressed and all had reached or exceeded a score of 3 at 48 h and were sacrificed. In contrast, five of six animals treated with a single i.v. dose (8 mg) of FB-28.4.2 at the time of disease induction had no detectable disease at 24 h (Fig. 7B) (no weakness or tail tone loss) and one had some tail tone loss (clinical score of 1); the treated group showed minimal weight loss (average 1.7% body mass). After 48 h, rats treated with mAb FB28.4.2 showed marginally increased signs of disease (three of six with clinical score of 1; Fig. 7B) and weight loss (Fig. 7A). All rats were sacrificed at 48 h to collect tissues and serum.

Complement hemolytic activity was measured in serum harvested from the rats prior to disease induction, then at 4, 24, and 48 h postinduction, using a specific AP assay (as described in *Materials and Methods*). Four hours after FB28.4.2 administration, plasma AP hemolytic activity was drastically reduced by 90% compared with predose and PBS controls. At 24 and 48 h, the hemolytic activity was still markedly reduced—by 70 and 50%, respectively (Fig. 7C).

To confirm the impact of the mAb on pathology at the end-plate in EAMG, intact Bungarotoxin-stained endplates were counted in Soleus muscle sections from control and treated rats. In control EAMG rats, intact endplates were rare, averaging 1.6 per field at $\times 20$ magnification; in contrast, endplates in FB28.4.2-treated animals were significantly more frequent, averaging 10.6 per field at $\times 20$ magnification (Fig. 7D).

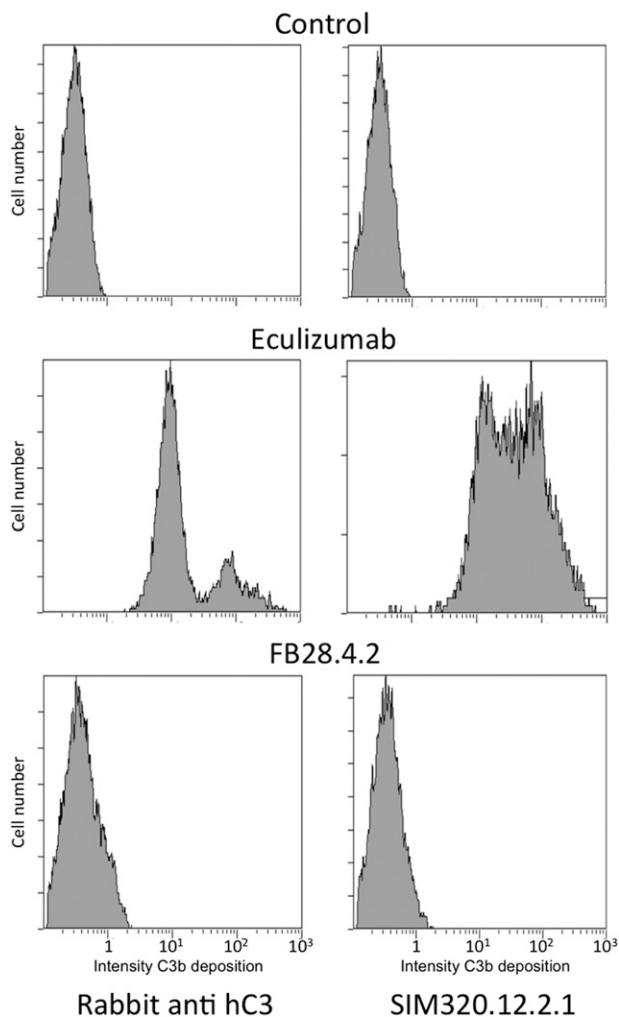


FIGURE 5. FB28.4.2 prevents C3 deposition on erythrocytes. Rabbit erythrocytes were incubated with NHS in the presence of eculizumab (20 μg) or FB28.4.2 (9 μg) at 37°C in AP buffer and analyzed by flow cytometry for the presence of C3 activated fragments using a rabbit polyclonal anti-C3 Ab or an anti-iC3b/C3dg mouse mAb (SIM320.12.2.1). As a negative control, rabbit erythrocytes were incubated with NHS in the presence of EDTA. This experiment was performed twice with identical results.

These data illustrate that FB28.4.2 efficiently blocks complement activation in vivo and prevents complement-mediated end-plate damage and the development of paralysis in EAMG, further supporting the large body of evidence indicating that EAMG is a complement-mediated disease and suggesting that AP inhibition is a potential therapy for the human disease.

Discussion

During the last 15 y, a number of linkage studies and genome-wide association studies have revealed a strong association between mutations and polymorphisms in complement genes and different diseases such as aHUS, C3 glomerulopathies, and AMD. Subsequent functional analyses of various disease-associated genetic variants have demonstrated that complement dysregulation is a major contributor to pathogenesis in these disorders (42). These findings, in addition to the long-time realization that unwanted complement activation sustains the “vicious cycle” of inflammation and perpetuates tissue damage in many pathological conditions, have put complement inhibition in focus for development of new therapeutic agents (16). Eculizumab, an

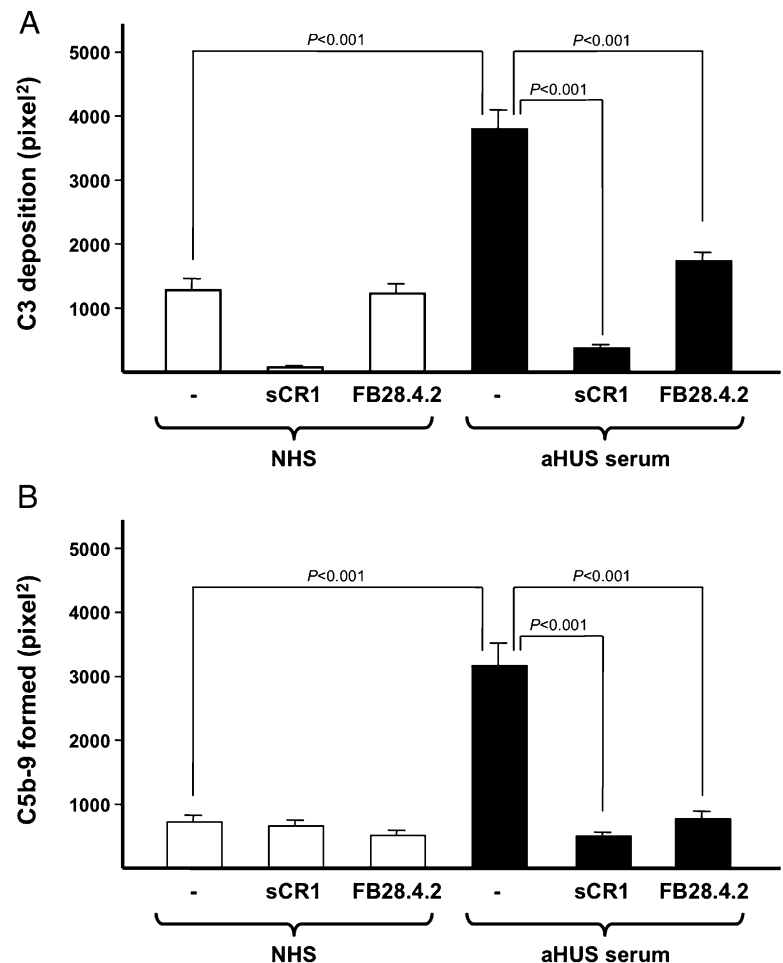


FIGURE 6. FB28.4.2 prevents C3 and C5b-9 deposition on endothelial cells exposed to serum from an aHUS patient. Activated HMEC-1 cells were incubated with NHS or serum from an aHUS patient carrying a *CFH* mutation, in the presence or absence of the complement inhibitor sCR1 (150 $\mu\text{g/ml}$) or FB28.4.2 (500 $\mu\text{g/ml}$). The cell surface area covered by C3 fragment (**A**) and C5b-9 (**B**) deposits was assessed by immunofluorescence staining and automated image analysis. The results represent the mean \pm SD of three independent experiments.

inhibitory Ab that block activation of C5 and initiation of the terminal pathway, is the first complement drug approved (43, 44), but others targeting different complement proteins are required to satisfy the urgent medical need for these treatments. Inhibitors of the AP are particularly interesting because the AP is critical for the efficient activation of complement regardless of the trigger that initiates complement activation. Current AP inhibitors under development include modified complement components, naturally occurring compounds, small synthetic molecules and inhibitory Abs (45). In this paper, we describe the generation of FB28.4.2, an inhibitory Ab targeting an evolutionary-conserved epitope in the Ba fragment of FB. FB28.4.2 shows high affinity (K_D , 3.55 nM) for human FB and efficiently inhibits formation of the AP C3 proconvertase by blocking the interaction between the Ba domain of FB and C3b.

Western blot analyses demonstrate that FB28.4.2 recognizes FB from a large number of mammals species, although there are likely important differences in the affinity values for the interaction between FB28.4.2 and the different FBs. A high affinity will explain, for example, why FB28.4.2 stains strongly human, rat, and pig FB and consequently works well blocking activation of the AP in these species. Indeed, hemolysis inhibition data generated using rabbit or sheep erythrocytes and human sera containing known concentrations of FB illustrated that both intact FB28.4.2 and its Fab fragment are capable of inhibiting lysis at concentrations approximately equimolar with FB. In contrast the affinity of FB28.4.2 for mouse FB is likely low, and the data show that it is not an efficient inhibitor of the AP in this species. Despite this limitation, we found mAb FB28.4.2

extraordinarily useful to deplete mouse serum from FB and to purify mouse FB by affinity chromatography. To our knowledge, there is only one additional mAb targeting FB that has been reported previously to inhibit AP activation. This previous mAb, termed 1379, also recognizing an epitope in the Ba fragment of FB and inhibiting formation of the AP C3 convertase, was generated immunizing mice with a recombinant protein containing the second and third short consensus repeats of mouse FB (10). The fact that mAb1379 targets primarily mouse FB and efficiently inhibits AP activity in mouse serum indicates that mAb1379 and FB28.4.2 are distinct mAbs, likely targeting different epitopes in Ba. Although both mAbs very efficiently inhibit the AP activity in serum from humans and other mammalian species, the potential advantages of FB28.4.2 over mAb1379 will require direct comparisons, which we have not been able to address.

To provide proof of concept of the usefulness of FB28.4.2 to prevent or ameliorate the consequences of complement activation in diseases characterized by AP complement dysregulation, we have used *ex vivo* models of PNH and aHUS and an *in vivo* rat model of MG. PNH patients are currently treated with eculizumab. This has improved significantly the quality of life of these patients. However, because eculizumab does not block the activity of the AP C3 convertase, C3 opsonization of PNH erythrocytes persists, resulting in variable percentages of erythrocytes covered by C3 fragments, which are susceptible to extracellular hemolysis (32, 33). Using PNH erythrocytes obtained from patients treated with eculizumab, we were able to show that FB28.4.2 efficiently prevented their lysis in the presence of

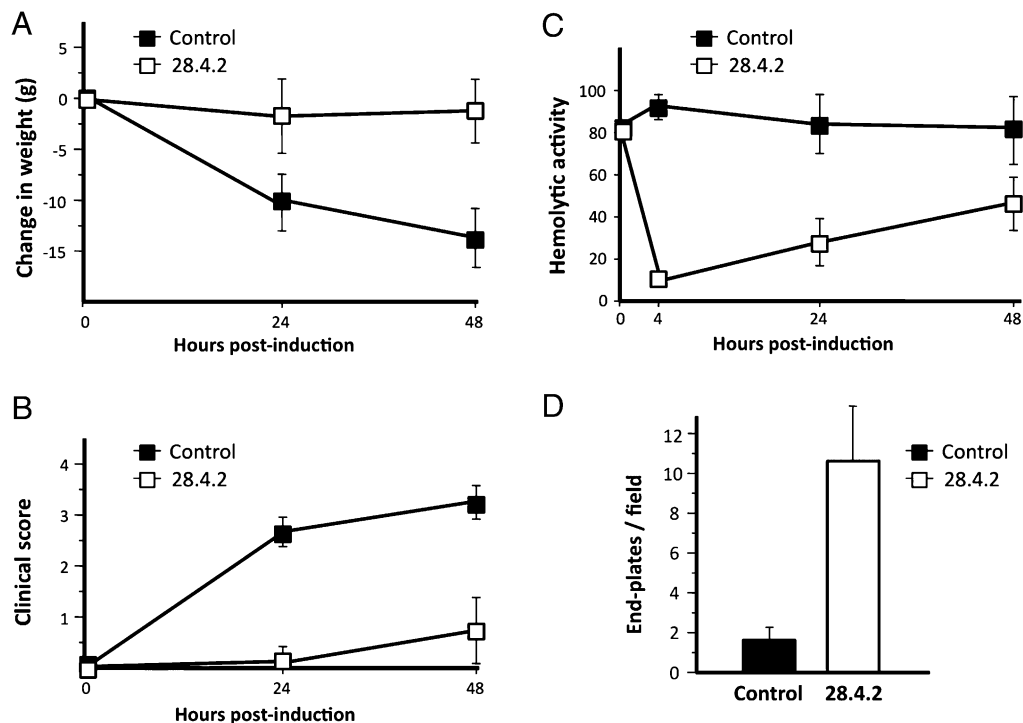


FIGURE 7. FB28.4.2 prevents development of EAMG in rats. After anti-AChR administration (time 0), change in weight (**A**), clinical score (**B**), and hemolytic activity (**C**) were monitored in both groups, control animals were injected i.v. with vehicle alone ($n = 6$), and animals were treated with FB28.4.2 ($n = 6$). The impact of the mAb on pathology was confirmed by counting the number of intact neuromuscular junctions (endplates) in bungarotoxin-rhodamine-stained Soleus muscle sections (average of 5 fields of view at $\times 20$ magnification; counted blind) from control and FB28.4.2-treated rats (**D**). The results represent the mean \pm SD of three independent experiments. Clinical score as follows: 0, can grip and lift lid of cage; 1, can grip but cannot lift; 2, cannot grip; 3, cannot grip + hind limbs paralyzed; and 4, moribund. All differences obtained from the comparison between using FB28.4.2 mAb and vehicle alone were statistically significant.

acidified serum. As expected, these PNH erythrocytes were covered by C3 fragments, which impeded our ability to demonstrate that FB28.4.2 would also be useful in preventing C3 opsonization of PNH erythrocytes. However, experiments performed with rabbit erythrocytes to compare the capacity of eculizumab and FB28.4.2 to inhibit complement-mediated lysis and C3 deposition strongly support the suggestion that FB28.4.2 would improve treatment of PNH by preventing both extravascular and intravascular hemolysis through the inhibition of C3 deposition and MAC formation, respectively.

aHUS is another disease in which the mAb FB28.4.2 could be used to restore the AP complement dysregulation associated with mutations in complement genes and to prevent the vascular endothelial damage caused by excessive terminal pathway activation. We have tested this possibility in a recently developed aHUS ex vivo model based on the capacity of ADP-activated HMEC-1 cells to activate the AP, which results in strong C3 fragment and C5b-9 deposition when these activated cells are exposed to serum from aHUS patients. In agreement with the conclusions from our experiments in the PNH model, when ADP-activated HMEC-1 cells were incubated with aHUS serum in the presence of FB28.4.2, C3 fragment and C5b-9 deposition was drastically reduced.

The experiments performed with PNH erythrocytes and HMEC-1 cells are in vitro experiments. To test whether the FB28.4.2 Ab is able to block the AP in vivo, we used the passive EAMG model in rats. We were able to show that a single i.v. dose of 8 mg FB28.4.2 blocked significantly the AP in the rat serum for at least 48 h, which in this acute animal model was sufficient to prevent the complement-mediated endplate damage and the development of hind-limb paralysis in the animals treated with the anti-AChR mAb35. As a whole, the data presented in this paper demon-

strate the potential value of FB28.4.2 for the treatment of diseases caused by complement dysregulation in humans and animal models.

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Disclosures

S.R.d.C., M.D., M.S., and O.L. are named on a patent held by the Agencia Estatal Consejo Superior de Investigaciones Científicas and the Instituto de la Salud Carlos III that protects the use of the mAb FB28.4.2 as a complement inhibitor. This situation has not influenced the results and interpretations in this article. The other authors have no financial conflicts of interest.

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