Non-inhibitory antibodies inducing increased emicizumab clearance in a severe hemophilia A inhibitor patient

Hemophilia A is a bleeding disorder that results from coagulation factor VIII (FVIII) deficiency, which can be treated via substitution therapy using FVIII concentrates. However, the generation of neutralizing antibodies renders treatment ineffective in up to 30% of the severe patients.[1,2] Emicizumab is a humanized bispecific antibody that binds simultaneously to activated factor IX (FIXa) and factor X (FX), thereby mimicking the cofactor function of activated factor VIII (FVIIIa), even in the presence of FVIII inhibitors.[3,4] Once-weekly subcutaneous administration of emicizumab markedly decreased the bleeding rate in patients who had hemophilia A with or without FVIII inhibitors.[5-8] However, anti-drug antibodies with neutralizing potential can develop in a small number of patients and have been associated with decreased emicizumab plasma concentrations and loss of efficacy.[9,10] Although the development of neutralizing anti-emicizumab antibodies is rare and routinely biological monitoring is not recommended in patients treated with emicizumab, it is still important to detect the presence of such antibodies in case of bleeding events. In this study, we describe the development of non-inhibitory anti-emicizumab antibodies that selectively provoke increased emicizumab clearance in a severe hemophilia A patient with inhibitors.

A 2-year-old boy with severe hemophilia A developed an anti-FVIII inhibitor (1 BU/mL) at 19 exposure days. The patient failed to respond to immune tolerance induction and venous access became extremely complicated.

Treatment with emicizumab was therefore initiated with four loading doses (3 mg/kg/week) followed by weekly treatment (1.5 mg/kg/week). Clinical outcomes were excellent with no bleeding episodes or bruising. Blood samples were taken as part of routine care, with excess being stored for research (The Hôpital Necker's hemophilia bio-library/Necker Biobank, registration number: DC-2009-955; procedure is in accordance with the Helsinki declaration and participants gave written informed consent). Analysis revealed emicizumab concentrations in the expected range (66 μg/mL 52 days after emicizumab initiation),[5] and a dramatic decrease in activated partial thromboplastin time (APTT) ratio (0.74, normal range <1.2) was measured (Figure 1).

A spontaneous hemarthrosis of the ankle occurred 6 months after emicizumab initiation, which was confirmed via clinical examination and ultrasound evaluation. Simultaneously, the APTT-ratio rose to 2.67, and circulating emicizumab concentrations were below 1 μg/mL (Figure 1). Hence, the development of anti-emicizumab antibodies was suspected, and the presence of emicizumab-specific immunoglobulin G (IgG) in the patient's serum was analyzed in immunosorbent assays, using normal serum and IgG-depleted patient serum as controls. Binding of IgG to immobilized emicizumab (5 μg/mL) was determined using isotype-specific peroxidase-labeled monoclonal antibodies. Whereas no specific IgG2 or IgG3 anti-emicizumab antibodies were detected, the patient's serum was indeed enriched in anti-emicizumab antibodies of the IgG1 subtype (Figure 2A and B). We could not test for IgG4 antibodies, since emicizumab is of this subtype.[3]

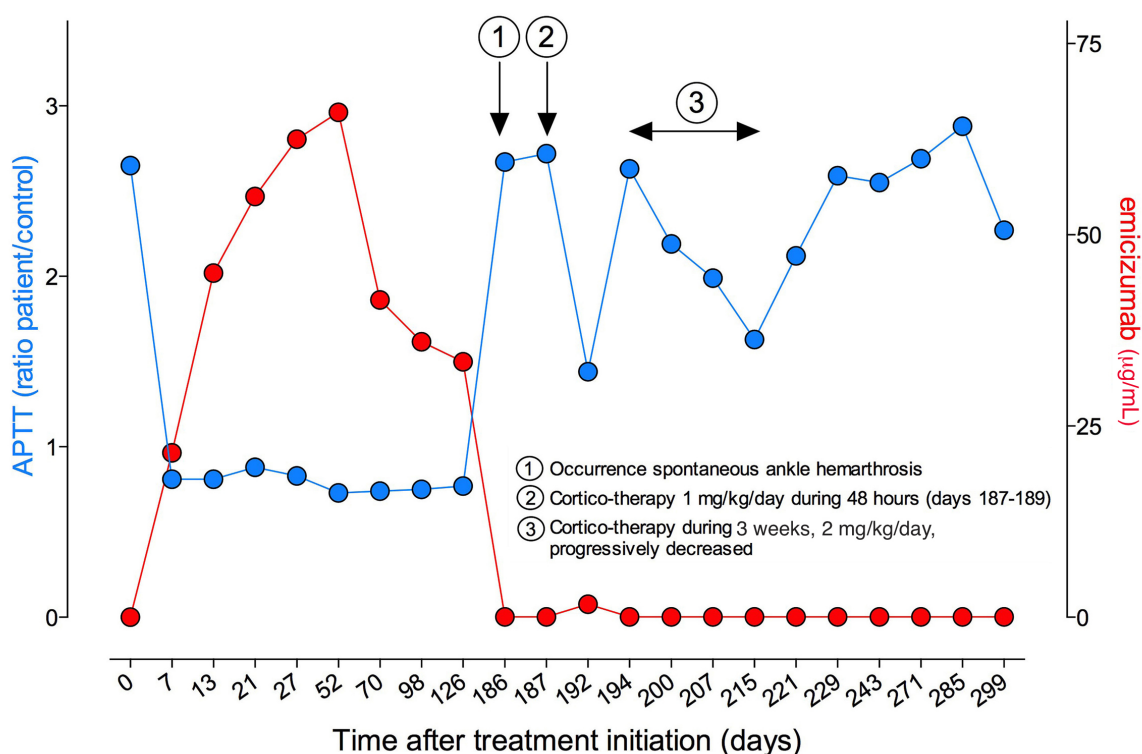Treatment of the hemarthrosis included rFVIIa and oral

Figure 1. Evolution of APTT and emicizumab plasma concentration over time. At indicated time points, blood samples were taken from the patient. Plasma was then analyzed for APTT (left Y-axis, blue circles) and emicizumab concentration (right Y-axis; red circles). Arrow 1 indicates bleeding event; arrows 2-3 indicate periods of cortico-therapy (2: 1 mg/kg/day for 48 hours during day 187-189; 3: 2 mg/kg/day during 3 weeks during days 194-214, with progressive decrease in dosing). ATPP: activated partial thromboplastin time.
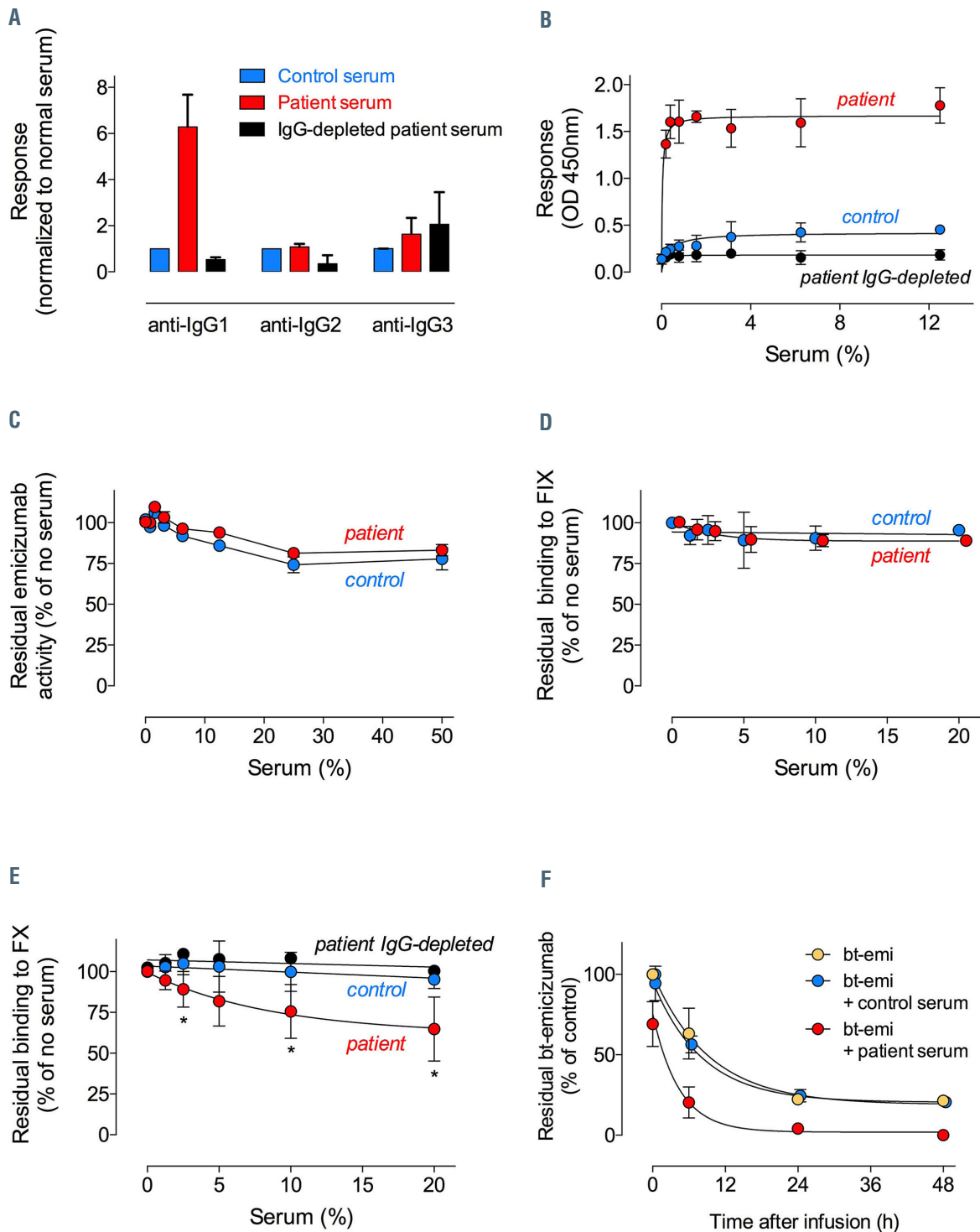
Figure 2. Characterization of anti-emicizumab antibodies. (A and B) Emicizumab was immobilized (5 μg/mL) and incubated with control serum (blue), patient serum (red) or immunoglobulin G (IgG)-depleted patient serum (black). Bound anti-emicizumab antibodies were probed using peroxidase-labeled IgG-subtype specific antibodies, and detected via 3,3',5,5'-tetramethyl benzidine (TMB) hydrolysis. For panel A, samples were diluted 256-fold, and response was normalized to that of normal plasma, which was arbitrarily set at 1. For panel B, the dose-response for binding of IgG1 antibodies to emicizumab is shown. (C) Emicizumab (25 μg/mL) was incubated in the absence or presence of various dilutions of control serum (blue circles) or patient serum (red circles). Presented is the percentage residual emicizumab activity relative to the absence of serum as measured in a chromogenic factor VIII (FVIII)-activity assay using human FIXa and factor X (FX). (D) Binding of bt-emicizumab (50 μg/mL) to immobilized factor IX (FIX) (5 μg/mL) was performed in the absence or presence of various dilutions of control serum (blue circles) or patient serum (red circles). Bound bt-emicizumab was probed with peroxidase-labeled streptavidin and detected via TMB hydrolysis. Shown is the percentage residual FIX binding relative to the absence of serum. (E) Binding of bt-emicizumab (10 μg/mL) to immobilized FX (5 μg/mL) was performed in the absence or presence of various dilutions of control serum (blue circles) or patient serum (red circles). Bound bt-emicizumab was probed with peroxidase-labeled streptavidin and detected via TMB hydrolysis. Shown is the percentage residual FX binding relative to the absence of serum. Statistical assessment was performed using multiple t-test analysis between control and patient serum. Stars indicate $P<0.05$ as analyzed in a multiple t-test comparing control serum and patient serum. (F) Immuno-deficient mice received bt-emicizumab (0.25 mg/kg) alone (orange circles) or in the presence of control serum (100 μL; blue circles) or patient serum (100 μL; red circles) via intravenous injection in the retro-orbital plexus. At indicated time points, samples were taken and plasma was analyzed for the presence of residual bt-emicizumab. Presented is the percentage of residual bt-emicizumab relative to bt-emicizumab alone at 3 minutes after injection, which was arbitrarily set at 100%. Lines were generated by fitting the data to an equation describing a single-exponential decay. Data represent the mean ± standard deviation of three experiments.

corticoids (1 mg/kg/day for 48 hours [h] to reduce peri-articular inflammation). A minor increase in emicizumab concentrations (1.7 μg/mL) and reduction in APTT-ratio (1.44) was observed (Figure 1), suggesting a potential cortico-sensitivity of the anti-emicizumab antibody-producing plasmocytes. Although no bleeds were observed during a 3-week period, emicizumab levels remained undetectable following a short corticosteroid therapy (2 mg/kg/day, conform to the management of children's immunologic thrombocytopenic purpura). Corticosteroid-therapy was therefore halted. Since anti-emicizumab antibodies have been reported to be transient in some patients,[11] emicizumab therapy (1.5 mg/kg/week) was continued for 3 months. As no improvement was observed, emicizumab therapy was terminated.

In order to further characterize the anti-emicizumab antibodies, additional tests were performed. We next analyzed eventual inhibition of emicizumab activity using the chromogenic activity assay (Biophen FVIII:C activity assay [ref 221402]; Hyphen BioMed, Andresy, France). Surprisingly, no reduction in emicizumab activity was observed, irrespective of whether normal or patient serum was tested (Figure 2C). Similar data were obtained using a one-stage clotting assay, suggesting that the anti-emicizumab antibodies are essentially non-inhibitory.

This was further assessed by analyzing binding of biotinylated (bt)-emicizumab to immobilized purified recombinant FIX (Pfizer, Paris, France) or plasma-derived FX (Cryopep; Montpellier, France). Biotinylation was performed using the NHS-PEO4-biotin kit (ref: UPR2027B; Griener Bio-one; Frickenhausen, Germany) without loss of cofactor activity. No inhibition of emicizumab binding to FIX was observed with normal or patient serum (Figure 2D). However, a modest inhibition of emicizumab binding to FX was observed using patient serum (Figure 2E). Maximal inhibition was 35±20% (n=4, P=0.0124 compared to normal serum) using 5-fold diluted serum. The absence of a dominant inhibitory effect on the interactions between emicizumab and FIX/FX is compatible with the non-inhibitory nature of the patient's anti-emicizumab antibodies in the activity assays. Indeed, a modest decrease in emicizumab-FX complex formation would still allow for sufficient ternary complex to be formed (555 pM *vs.* 851 pM in the absence of inhibitor, when calculated according to Kitazawa *et al*.).[12] We previously showed that a threshold of about 300 pM ternary complex is needed to produce sufficient hemostatic activity *in vivo*.[13]

As low emicizumab levels in the patient can be explained by an increased clearance, we determined survival of bt-emicizumab in the absence or presence of serum in immuno-deficient mice. Bt-emicizumab was added to 0.9% NaCl, 100 μL control serum or 100 μL patient serum (both 5 μg per 100 μL), and incubated for 30 minutes (min) at ambient temperature. Mixtures were then injected via the retro-orbital plexus (at a dose of 0.25 mg/kg bt-emicizumab) to male NOD.CB17-Prkdcscid/NCrHsd-mice (age 9 weeks; ENVIGO, Gannat, France). Housing and experiments of NOD.CB17-Prkdcscid/NCrHsd-mice were performed in accordance with French regulations and the experimental guidelines of the European Community. Experimentation was approved by the local ethical committee of the Université Paris-Sud (Comité d'Éthique en Experimentation Animale no. 26, protocol APAFIS#4400-2016021716431023v5). Blood samples were taken at 15 min, 6 h, 24 h and 48 h after infusion. Plasma was used to determine residual bt-emicizumab levels in an enzyme-linked immonosorbent assay (ELISA) in which streptavidin-coated microtiter wells were incubated with plasma samples, and bound bt-emicizumab was probed using peroxidase-labeled anti-human IgG4-Fc antibodies. No difference in the disappearance from the circulation was found between bt-emicizumab alone or the bt-emicizumab/normal serum combination ($t_{1/2}$=8.5 h and 8.3 h, respectively; Figure 2F). In contrast, bt-emicizumab was eliminated significantly faster in the presence of patient serum ($t_{1/2}$=2.2 h; P=0.0013; Figure 2F), with no bt-emicizumab detectable at 48 h after infusion (compared to 21% for both other conditions). It, thus, seems that the main effect of the anti-emicizumab antibodies is to accelerate clearance of emicizumab.

Contrary to previously reported inhibitors of emicizumab, we here describe the presence of antibodies that leave emicizumab activity unaffected, but that provoke the rapid elimination of emicizumab in mice, providing an explanation for the low emicizumab levels in the patient. Although the occurrence of anti-emicizumab antibodies is rare, their presence may severely diminish its clinical efficacy, resulting in the re-appearance of spontaneous bleeds. Clinical monitoring will usually be sufficient for patients receiving emicizumab therapy. However, in case of bleeding in the absence of any compliance concerns, biological monitoring (APTT and emicizumab concentration) is therefore helpful to detect possible anti-drug antibodies.

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