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ORIGINAL ARTICLE

Haemostasis

Detailed analysis of anti-emicizumab antibody decreasing drug efficacy, using plasma samples from a patient with hemophilia A

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Abstract

Background: Emicizumab is a humanized bispecific monoclonal antibody that bridges activated factor IX (FIXa) and factor X (FX) to mimic the function of factor VIII (FVIII). It suppresses the bleeding tendency in hemophilia A patients with or without FVIII inhibitors. A case of an adult FVIII inhibitor-positive hemophilia A patient in whom treatment with emicizumab was discontinued owing to the repeated bleeding events and prolonged activated partial thromboplastin time.

Objective: To analyze the mechanisms of decreased efficacy of emicizumab.

Methods: Residual plasma samples were used to measure the following: emicizumab concentration in plasma, measured by enzyme-linked immunosorbent assay; titer of anti-drug antibody (ADA) against emicizumab, measured by electrochemiluminescence; and neutralizing activity against emicizumab, measured by Bethesda method modified by using emicizumab-spiked FVIII-deficient plasma.

Results: At week 31, emicizumab concentration was 15.0 μg/ml, and ADAs were measured as positive. Emicizumab concentration continued to decrease until emicizumab discontinuation point at week 49, and after week 50, emicizumab concentrations were below the limitation of quantification. The ADA titer increased transiently from week 31, even past the emicizumab discontinuation point at week 49. The ADA titer then gradually decreased until the last sampling point at week 93. Neutralizing activity against emicizumab was detected after emicizumab discontinuation. Epitope analysis showed that the ADAs recognize the anti-FIXa and anti-FX Fab arms of emicizumab, but not the Fc region.

Conclusion: The appearance of ADAs with emicizumab-neutralizing activity and potential to accelerate emicizumab clearance decreased the efficacy of emicizumab.

KEYWORDS
emicizumab, factor VIII, hemophilia A, hemostasis, neutralizing antibodies

Kaneda and Kawasaki are co-first authors and contributed equally Manuscript handled by: Ton Lisman

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1 | INTRODUCTION

Hemophilia A (HA) is characterized by a congenital deficiency or a functional decline in clotting factor VIII (FVIII). Disease severity depends on the level of this clotting factor in plasma (severe, <1%; moderate 1% to 5%; mild, >5% to <40%).[1,2] In the management of patients with HA, the main objective is the prevention of the bleeding that leads to a decline in the patients' quality of life. Emicizumab is a recombinant humanized bispecific monoclonal antibody that bridges activated clotting factor IX (FIXa) and clotting factor X (FX) and mimics the function of FVIII.[3,4] Unlike FVIII, emicizumab forms a tenase complex with FIXa and FX without requiring activation by thrombin.[4,5] Its hemostatic efficacy has been confirmed in several clinical studies.[6–10]

Immunogenicity is the ability of a therapeutic protein to provoke an undesirable immune response against that therapeutic protein. Anti-drug antibodies (ADAs) can develop after a single dose or repeated administration of a therapeutic protein.[11] The development of ADAs after the administration of a therapeutic protein may impact the pharmacokinetics, pharmacodynamics, efficacy and/or safety of the therapeutic protein.[12] Emicizumab is approved for the routine prophylaxis of bleeding episodes in HA patients with or without FVIII inhibitors. Although efforts were made to reduce the potential immunogenicity risk to the extent possible, emicizumab still has the potential for immunogenicity, as do all therapeutic proteins.[5] In Phase III clinical trials, ADAs were detected in 14 of 398 patients (3.5%), including 3 patients with ADAs showing neutralizing potential.[13] In addition, there were 3 cases who had been discontinued the administration of emicizumab. It was reported that neutralizing activity or rapid clearance from the blood was the causes of decrease in the efficacy which led to discontinuation of emicizumab.[14–16]

At Sapporo Tokushukai Hospital in Japan, there was an adult FVIII inhibitor-positive HA patient in whom treatment with emicizumab was discontinued 49 weeks from the initiation of emicizumab administration owing to increased bleeding frequency and prolonged activated partial thromboplastin time (APTT). Because of these signs, we suspected a decrease in the efficacy of emicizumab caused by the appearance of ADAs.

In this study, we investigated whether ADAs with the potential to decrease the efficacy of emicizumab was present in the patient's plasma. Having detected ADAs in the plasma, we then analyzed the characteristics of ADAs in detail, focusing on its neutralizing activity by using a one-stage clotting assay modified to account for the effect of emicizumab. In addition, we carried out the epitope analysis to investigate the regions of emicizumab to which ADAs bind.

Essentials

- Emicizumab (Emi) treatment was discontinued in a hemophilia A patient due to decreased efficacy.
- We measured the plasma Emi concentration and analyzed for anti-drug antibodies (ADAs).
- The decrease in Emi concentration and ADAs with neutralizing activity against Emi were observed.
- ADAs with neutralizing activity and potential to accelerate clearance decreased the efficacy.

2 | MATERIALS AND METHODS

2.1 | Patient

The HA patient was a 62 years old man with inhibitors to FVIII. Immediately prior to the initiation of emicizumab treatment, his APTT was prolonged (>240 s). He received the standard 3 mg/kg loading doses of emicizumab once weekly for 4 weeks and then proceeded to 1.5 mg/kg once weekly maintenance doses of emicizumab. Initially, emicizumab shortened the APTT and there was no bleeding. However, at 41 weeks from emicizumab treatment, the patient presented with an increase in bleeding. At 49 weeks into treatment, emicizumab was discontinued owing to increased bleeding frequency and prolonged APTT. Thereafter, bypass therapies were conducted when bleeding episodes were observed except at 43 weeks. Plasma samples had been periodically collected for the routine measurement of APTT, and the residual plasma samples were used for the measurements conducted in this study. The study was approved by the Tokushukai Group Ethics Committee and the Chugai Ethics Committee, and the patient signed informed consent forms.

2.2 | Materials

Recombinant emicizumab was produced using a Chinese hamster ovary cell line and recombinant antibodies for epitope analysis were produced using Expi293 Expression System (Thermo Fisher Scientific Inc.). A mouse antibody specific to the anti-FIXa Fab arm of emicizumab (rAQ8-mIgG2b) and a rabbit antibody specific to the anti-FX Fab arm of emicizumab (rAJ540-rbtIgG) had been prepared previously.[17] FVIII-deficient plasma collected from a single hemophilia A donor (George-King Bio-Medical Inc.), FVIII-deficient plasma (Sysmex), control plasma from each of nine normal individual donors (BioIVT), and Thrombocheck APTT-SLA (Sysmex) were purchased from the indicated vendors. Imidazole and hydrochloride (HCl) were purchased from Sigma-Aldrich Co. LLC.

2.3 | Measurement of plasma emicizumab concentration by using bridging ELISA

Bridging ELISA using anti-idiotype monoclonal antibodies can be used to measure the concentration of emicizumab specifically in human plasma.[18] The plasma emicizumab concentration was measured by bridging ELISA, using rAJ540-rbtIgG as the solid phase antibody to capture emicizumab and rAQ8-mIgG2b as the detecting antibody; horseradish peroxidase-conjugated goat anti-mouse

IgG (H+L) (Southern Biotechnology Associates, Inc.) was used as the enzyme-linked secondary detection antibody. For detection, ABTS Solution (Roche Diagnostics K.K.) was applied, followed by adding ABTS Peroxidase Stop Solution (SeraCare Life Sciences Inc.). Absorbance was measured at 405 nm (reference wavelength of 490 nm) using a microplate reader (SPECTRA max PLUS 384; Molecular Devices, LLC.).

2.4 | Detection of anti-emicizumab antibodies using electrochemiluminescent bridging immunoassay

Anti-drug antibodies (ADAs) against emicizumab in the plasma samples were detected by an electrochemiluminescent (ECL) bridging immunoassay.[18] Ten-fold diluted plasma samples from the HA patient were incubated with a solution containing 0.5 μg/ml biotin-labeled emicizumab and 1 μg/ml ruthenium-labeled emicizumab, and then the immune complexes, which form in proportion to the concentration of ADAs, were captured on a streptavidin-immobilized plate and detected with an electrochemiluminescence detector (Meso Scale Diagnostics, LLC.). A confirmation test was performed to check whether each screened ADA-positive sample had a specific response to emicizumab by ascertaining whether the addition of 10 μg/ml emicizumab to the plasma samples inhibited the binding of anti-emicizumab alloantibodies to the two types of emicizumab-labelled molecules.

2.5 | Measurement of neutralizing activity against emicizumab by a modified Bethesda method assay using emicizumab-spiked FVIII-deficient control plasma

The neutralizing activity was measured using a modified Bethesda method assay carried out on an automated coagulation analyzer (CS-2400; Sysmex).[19,20] Plasma samples from the patient and FVIII-deficient plasma (George King Bio-Medical, as the control plasma) were centrifuged at 20 000 *g* for 2 min at room temperature after heating at 56°C for 30 min. Buffered FVIII-deficient plasma (B-FVIII DP) was prepared by mixing FVIII-deficient plasma (Sysmex) and 5 M imidazole solution. The final B-FVIII DP contained 0.1 M of imidazole, and pH was adjusted to 7.4 ± 0.4 by using 1 N HCl. Samples for calibration curves were prepared by adding a series of concentrations of emicizumab (from 0.2 to 10 μg/ml or from 0.25 to 1 μg/ml) to the B-FVIII DP, then an equal amount of FVIII-deficient plasma (George King Bio Medical) heat-treated in the same way as the samples was added. To measure the neutralizing activity of the ADAs, B-FVIII DP was first spiked with emicizumab (concentrations were 5 and 0.5 μg/ml), and equal amounts of plasma samples were mixed with emicizumab-spiked B-FVIII DP. Calibration samples and measuring samples were then incubated at 37°C for 2 h. Clotting times of samples were recorded using the one-stage clotting assay protocol, then emicizumab concentration was calculated using the calibration curves. Neutralizing activity (%) was evaluated using calculated emicizumab concentration with the following equation: 100 − (emicizumab concentration of patient's sample / emicizumab concentration of control samples (5 or 0.5 μg/ml) × 100). In addition, Bethesda titer was calculated using previously described method[21] with modification. Previous method converted % residual FVIII activity to Nijmegen-Bethesda unit (NBU). Since our study measured the residual emicizumab concentration of spiked emicizumab, we substituted % residual FVIII activity to % residual emicizumab concentration, which was calculated by: emicizumab concentration of patient's sample / emicizumab concentration of control sample (5 μg/ml) × 100. Modified NBU was calculated by following equation: (2 − log%residual emicizumab concentration) / log2.

2.6 | Epitope analysis of anti-emicizumab antibodies

Epitope analysis was conducted for mechanistic identification of the regions of emicizumab that bind to the anti-emicizumab antibodies in the HA patient-derived plasma samples or to the animal-derived anti-emicizumab monoclonal antibodies (rAQ8-mIgG2b and rAJ540-rbtIgG). The method of epitope analysis was designed on the basis of the ECL bridging immunoassay described above for detection of anti-emicizumab antibodies. Several recombinant antibodies were engineered, each of which was designed to contain a specific functional region of emicizumab (Figure S1). Two plasma samples collected from the HA patient at 69 weeks from the initiation of emicizumab treatment (20 weeks after emicizumab discontinuation) and at 45 weeks from the initiation of emicizumab treatment (4 weeks before emicizumab discontinuation) or control plasma containing rAQ8-mIgG2b and rAJ540-rbtIgG were ten-fold diluted with a solution containing each competitive antibodies (10 μg/ml of each antibody and emicizumab, 3.5 μg/ml of Ab8 [emicizumab Fc region molecule, molar concentration equal to that of emicizumab]). These mixtures were incubated with the two types of labeled emicizumab molecules, in which the competitive binding between the recombinant antibody and labeled emicizumab molecules in the plasma attenuates the electrochemiluminescence signal intensity. Subsequent procedures were followed the method described above for detection of anti-emicizumab antibodies.

3 | RESULTS

3.1 | APTT and bleeding events of the HA patient from initiation of emicizumab treatment

An HA patient with inhibitors was started on emicizumab treatment at Sapporo Tokushukai Hospital. The normal range of APTT was from 25 to 40 s. The patient's APTT was >240 s before the

initiation of emicizumab treatment. APTT was shortened to <25 s after moving onto a 1.5 mg/kg once weekly emicizumab regimen, and no bleeding symptoms were observed. At week 39, APTT was 27.8 s. At week 41, the first bleeding episode was observed. Emicizumab was discontinued at week 49 owing to prolonged APTT (92.6 s) and repeated bleeding (Treated bleed: joint bleeding at week 41; nose bleeding at weeks 44 and 46; left erector spine muscle hemorrhage at week 49, untreated bleed: nose bleeding at week 43) (Figure 1). Residual plasma samples from the patient from before the initiation of emicizumab treatment and from 31 weeks from the initiation of emicizumab treatment had been stored. We used those residual plasma samples in the following measurements.

3.2 | Measurement of emicizumab concentration and ADA titer in the HA patient-derived plasma

A decrease in plasma emicizumab concentration was assumed to account for these unexpected bleeding symptoms, and we measured the plasma concentration of emicizumab. The concentration of emicizumab in the plasma was 15.0 μg/ml at week 31 and continued to decrease thereafter. At the point of emicizumab discontinuation, the concentration of emicizumab in the plasma was 0.0876 μg/ml (Figure 2A). Because we suspected the appearance of anti-drug antibodies (ADAs) against emicizumab to be the cause of the decrease in the concentration and efficacy of emicizumab, we measured ADAs using the ECL method. ADAs were present in the plasma from week 31, and the ECL signals derived from the ADAs continued to increase in subsequent samples. These ECL signals continued to increase after the discontinuation of emicizumab (week 49) until week 57, and then decreased gradually (Figure 2B).

3.3 | Measurement of emicizumab-neutralizing activity in the HA patient-derived plasma

Next, to investigate whether the ADAs had neutralizing activity against emicizumab, we used a modified Bethesda method using FVIII-deficient control plasma spiked with 5 or 0.5 μg/ml emicizumab. Before the discontinuation of emicizumab, residual emicizumab in the patient's plasma might have influenced and no apparent neutralizing activity was observed (Figure 3A,B). The neutralizing activity of ADAs became apparent after discontinuation of emicizumab and it showed maximum inhibition at 57 weeks from the initiation of emicizumab treatment. The neutralizing activity was present until 93 weeks from the initiation of emicizumab (44 weeks after the discontinuation of emicizumab treatment) in measurements using the FVIII-deficient control plasma spiked with 0.5 μg/ml emicizumab (Figure 3B).

3.4 | Examination of binding of ADAs in the HA patient-derived plasma to the FIXa or FX Fab arm of emicizumab

Since we had demonstrated the presence of ADAs with potential to neutralize the effect of emicizumab in the plasma samples from the HA patient, we next investigated which region of emicizumab is recognized by the ADAs. To address this challenge, we designed a competitive binding experiment based on the method of the ECL bridging immunoassay. Emicizumab, anti-FIXa monospecific antibody (Ab3; Figure S1), or anti-FX monospecific antibody (Ab4; Figure S1) was added to the plasma samples prior to incubation with biotin- and ruthenium-labeled emicizumab. Binding of ADAs to the specific region of emicizumab contained in the competitor antibodies interferes with formation of the ADA-labeled-emicizumab complex, resulting in attenuation of the ECL signal.

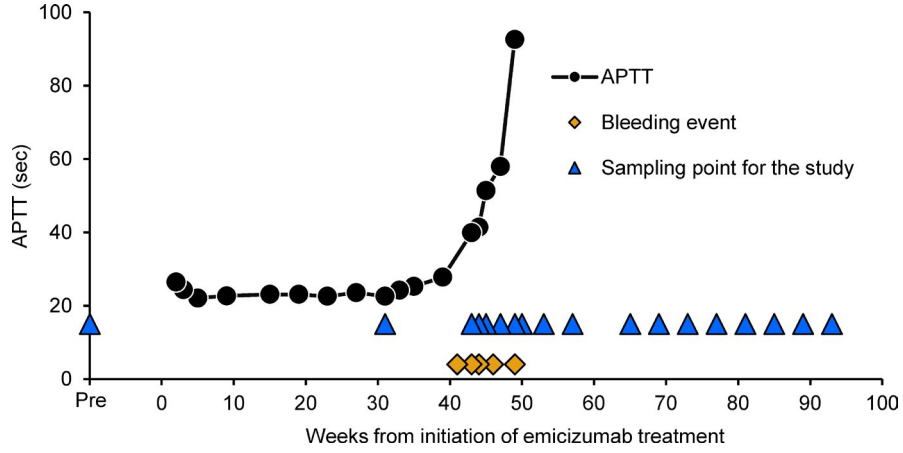

FIGURE 1 Changes in APTT and bleeding events in the HA patient from initiation of emicizumab treatment. Plasma samples were collected from the hemophilia A patient to measure APTT periodically. APTT (black circles), bleeding event (orange diamonds), collecting points of plasma samples (blue triangles) are shown. The patient's APTT was >240 s before the initiation of emicizumab treatment. Emicizumab treatment was discontinued at week 49 [Color figure can be viewed at wileyonlinelibrary.com]

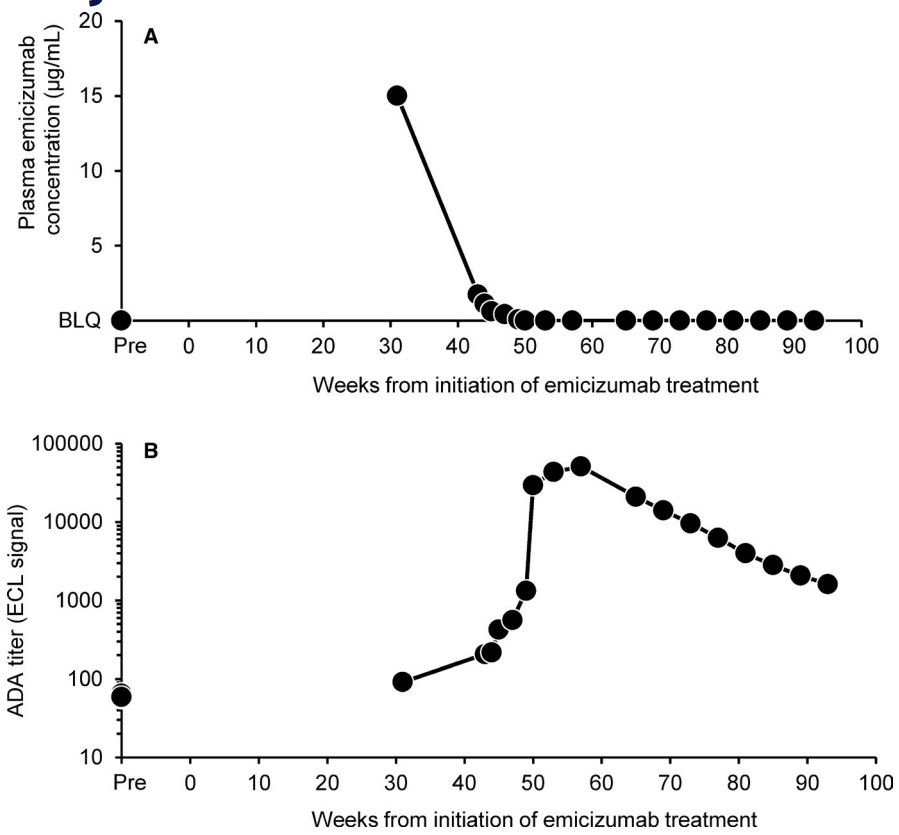

FIGURE 2 Changes in emicizumab concentration and expression of anti-emicizumab antibodies (ADAs) in plasma samples collected from the HA patient. (A) All plasma samples collected from the HA patient were subjected to the bridging ELISA using two types of the anti-idiotype monoclonal antibody for measurement of the concentration of emicizumab specifically in human plasma. Values of samples after week 50 were below the limit quantitation (BLQ). Data are shown as means of duplicate measurements. (B) Each plasma sample collected from the HA patient was ten-fold diluted and incubated with two types of labeled emicizumab molecules prior to detection of an electrochemiluminescent (ECL) signal. Emicizumab treatment was discontinued at week 49. Data are shown as means of duplicate measurements

Among the HA patient's plasma samples collected from weeks 31 to 93, which were all judged to be ADA-positive, treatment of emicizumab decreased the ECL signal (Figure 4). Following treatment with emicizumab-derived anti-FIXa monospecific antibody and emicizumab-derived anti-FX monospecific antibody, the respective ECL signals were decreased in ADA-positive plasma samples, which indicated that the plasma sample from the HA patient contains ADAs binding to either anti-FIXa monospecific antibody or anti-FX monospecific antibody.

3.5 | Epitope analysis of ADAs in the HA patient-derived plasma

Next, to investigate the epitope of patient-derived ADAs from the perspective of the binding regions, we prepared several other recombinant antibodies and emicizumab Fc region molecules (Figure S1) for use as competitors in the ADA detection assay. First, to confirm the reactivity and specificity of these competitor antibodies, we performed epitope analysis using animal-derived antibodies recognizing the anti-FIXa arm of emicizumab (rAQ8-mIgG2b: rAQ8) and anti-FX arm of emicizumab (rAJ540-rbtIgG: rAJ540) (Table S1 and S2). rAQ8 specifically bound to emicizumab via the complementarity-determining region 2 (CDR2) in the heavy chain of its anti-FIXa arm, but not the light chain or Fc region, and rAJ540 bound to the whole Fab molecule (the combination of heavy chain and light chain) of emicizumab, but not the Fc region (The details of results were shown in Supporting Information). These data thus indicated that this approach is functional for distinguishing the binding site of ADAs against emicizumab.

We then performed epitope analysis using two types of ADA-positive plasma: one collected from the HA patient before discontinuation of emicizumab (week 45) and one collected after (week 69) (Table S3, Table 1, respectively). With the plasma sample collected after emicizumab discontinuation, the ECL signals relative to that from plasma treated with no antibodies as 100% were 22% with anti-FIXa monospecific antibody (Ab3) treatment and 59% with anti-FX monospecific antibody (Ab4) treatment. The signal of the plasma sample treated with the mixture of Ab3 and Ab4 was 0%, and those with emicizumab (Ab1) was 1% (Table 1). These data indicated that all ADAs detected in this assay bound to either anti-FIXa monospecific antibody or anti-FX monospecific antibody. The relative ECL signal derived from the plasma treated with the emicizumab-Fc-region-molecule (Ab8) was 98%, indicating that the ADAs did not bind to the Fc regions. Furthermore, the relative ECL signals treated with the antibody consisting of the negative control heavy chain and emicizumab common light chain (Ab5), emicizumab-FIXa arm heavy chain and negative control light chain (Ab6), and emicizumab-FX arm heavy chain and negative control light chain (Ab7) were 88%, 95%, and 94% respectively, indicating that the whole Fab molecule (the combination of heavy chain and light chain) of emicizumab was essential for binding of ADAs because the binding property of ADAs was lost by replacing emicizumab's heavy chain or light chain into the negative control's sequence. In addition, even though the ECL signals from plasma sampled before emicizumab discontinuation (week 45) were lower than those of the plasma sampled after emicizumab

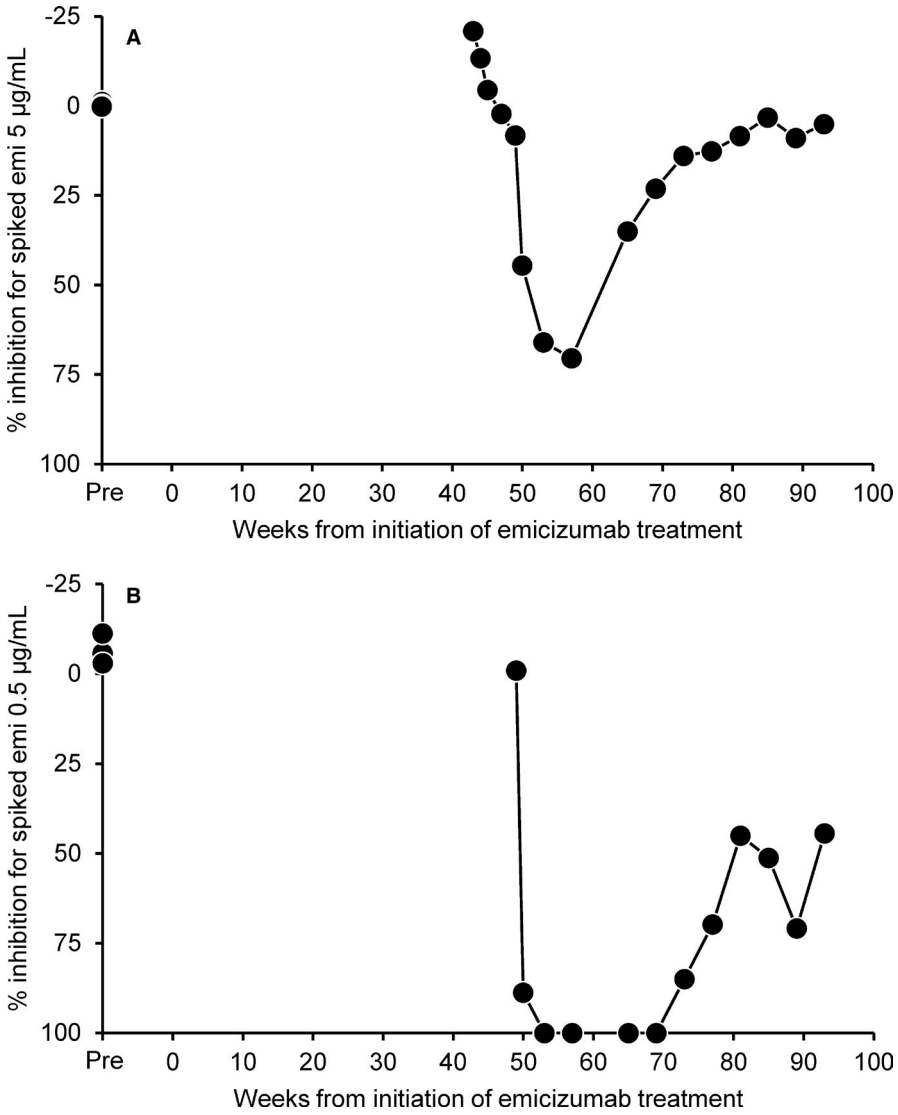

FIGURE 3 Measurement of neutralizing activity of ADAs with a modified one-stage clotting assay. (A, B) The neutralizing activity of ADAs was measured using FVIII-deficient control plasma spiked with 5 μg/ml emicizumab (A) or 0.5 μg/ml emicizumab (B). (A) Neutralizing activity was detectable at each point at which emicizumab concentration in the spiked control plasma fell below 5 μg/ml. The value at week 31 was above the calibration curve range. (B) Neutralizing activity was detectable at each point at which emicizumab concentration in the spiked control plasma fell below 0.5 μg/ml. The values at weeks 31, 43, and 44 were above the calibration curve range. Emicizumab treatment was discontinued at week 49. Data are shown as means of triplicate measurements

discontinuation, the patterns of competition were similar (Table S3). From these data, we elucidated that both the heavy chain and light chain consisting of the FIXa- and FX-binding Fab region of emicizumab are necessary for interaction with the ADAs in this study.

4 | DISCUSSION

In this report, we showed that ADAs against emicizumab appeared in a patient undergoing treatment with emicizumab at Sapporo Tokushukai Hospital, and that the concentrations of emicizumab in the patient's plasma fell to levels not sufficient to prevent bleeding, necessitating discontinuation of emicizumab treatment. In addition, by means of a one-stage clotting assay with modifications to account for the effect of emicizumab, we demonstrated that the ADAs had emicizumab-neutralizing activity.

From the previous clinical studies, the mean trough levels of emicizumab in plasma concentration at clinically therapeutic doses have been estimated to be approximately 30 μg/ml.[6–10] With regard to the patient at Sapporo Tokushukai Hospital, the plasma level of emicizumab was under 30 μg/ml at 31 weeks into emicizumab treatment, in which this level was below the trough concentration of emicizumab showed in clinical studies (Figure 2A). ADAs were detected in samples taken before the first bleeding event was observed, and the decrease in plasma emicizumab concentration was linked to the increase in the ECL signal derived from ADAs, suggesting that the appearance of ADAs could lead to the decrease in the efficacy of emicizumab (Figure 2B).

There are two types of mechanisms of ADAs on reducing a drug efficacy; one is directly blocking and interfering with the drug's binding to its target, and the other is accelerating drug clearance from the circulation.[12] In this study, we used the Bethesda method with modifications to account for the effect of emicizumab and demonstrated the ADAs in this patient having the neutralizing activity against emicizumab. It is suggested that neutralizing activity observed from the ADAs is partial because our data showed only about a half level of reduction in 5 μg/ml spiked emicizumab activity by ADAs, in which the spiked emicizumab concentration is one sixth of 30 μg/ml, the mean trough concentration of emicizumab in plasma. The result of epitope analysis indicated that ADAs in the

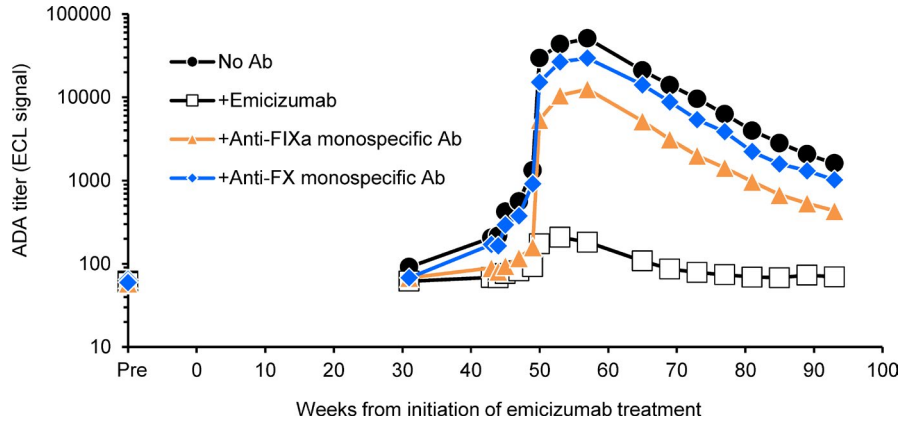

FIGURE 4 Examination of binding of ADAs in the HA patient-derived plasma to the FIXa or FX Fab arm of emicizumab. All plasma samples collected from the HA patient were ten-fold diluted and treated with 10 μg/ml of emicizumab, anti-FIXa monospecific antibody (Ab3 shown in Figure S1), or anti-FX monospecific antibody (Ab4 shown in Figure S1). Subsequent incubation with two types of labeled emicizumab molecules was followed by detection of an electrochemiluminescent (ECL) signal. Emicizumab treatment was discontinued at week 49. Data are shown as means of duplicate measurements [Color figure can be viewed at wileyonlinelibrary.com]

patient did not bind to Fc region but the FIXa-binding Fab region and FX-binding Fab region of emicizumab. We thus suggest that the ADAs can interfere with binding of emicizumab to FIXa and FX. On the other hand, as this characteristic of the ADAs alone cannot explain the weak neutralizing activity observed in a one-stage clotting assay, we speculate that an increased clearance of emicizumab simultaneously occurred with the interference with the emicizumab's binding to FIXa/FX by the ADAs, which finally caused a reduction in efficacy of emicizumab in the patient.

Valsecchi et al. investigated the biochemical characterization of a neutralizing ADA that developed in an HA patient who participated in the HAVEN 2 clinical trial and reported that the ADA mainly targeted the Fab region of emicizumab with a small amount of binding to the Fc region.[14] In addition, recently Harroche et al. examined anti-emicizumab antibodies that developed in a HA patient with inhibitors, and showed no inhibition of emicizumab binding to FIX and a modest inhibition of emicizumab binding to FX, but there was no reduction in emicizumab activity using a chromogenic activity assay and a one-stage clotting assay.[16] Although the measurement principles are different, our results showed that the ADAs in our patient did not bind to the Fc region but Fab region of emicizumab, and ADAs had a neutralizing activity against emicizumab. These results indicate that the ADA population can be different for each patient. Further analysis with a large number of patients is needed to determine whether there are particular regions of emicizumab that are highly likely to induce immunogenicity.

With regard to measurements of ADA titer and neutralizing activity, we point out that residual emicizumab could interfere with these measurement parameters. Therefore, the values we got from the result of ADA titer and neutralizing activity in the plasma might be lower than their true values due to the preexisting emicizumab-ADA complex, and indeed these measurement parameters showed a temporal increase immediately after the discontinuation of emicizumab (Figure 2B, Figure 3). Additionally, neutralizing activity was measured by the method based on one-stage clotting assay in our study, while Harkins Druzgal et al. previously measured neutralizing activity with the method based on chromogenic assay.[15] Considering the difference in the measurement principle between one-stage clotting assay and chromogenic assay, the influence of residual emicizumab on the measurement results might also be changed. Therefore, it is expected that measuring neutralizing activity using both methods would lead to a better understanding of the interference of residual emicizumab. From these observations, we indicate that carefully monitoring should be required in case of the measurements of ADA titer and neutralizing activity using plasma samples collected from the patients under emicizumab treatment, because residual emicizumab in the plasma may affect the measurement result. In order to deal with this, it could be helpful to remove residual emicizumab from the plasma by affinity purification.

In our study, neutralizing activity was calculated based on the measured residual emicizumab concentration of spiked emicizumab. When spiked emicizumab was 5 μg/ml, the maximum neutralizing activity of 70.5% was detected at week 57. At this point, modified Bethesda titer was calculated to be 1.76 Bethesda unit (BU). Harkins Druzgal et al. previously reported that a patient who developed a neutralizing ADA to emicizumab showed emicizumab inhibitor level of 1.98 BU at first laboratory test.[15] They used patients' plasma containing therapeutic level of emicizumab as FVIII deficient control plasma. Assuming the plasma emicizumab concentration would be over 30 μg/ml, 1.98 BU would result the neutralization of more than 20 μg/ml of plasma emicizumab. Therefore, even though the Bethesda titer between these patients were similar, emicizumab inhibitor level may have been higher in previously reported patients than the patient in this study.

In this patient, APTT slightly prolonged for 5.7 s just before the first bleeding was observed compared to week 5 when the 1.5 mg/kg once weekly treatment regimen was started, and then continued to lengthen with the repeated bleeding (Figure 1). A reduction in the efficacy of emicizumab may be suspected if APTT continues to

TABLE 1 Epitope analysis of ADAs in the HA patient-derived plasma at week 69 (20 weeks after emicizumab discontinuation)

Ab No.	ECL signal	Relative signal (%) compared to (-)	Heavy chain 1		Heavy chain 2		Light chain
			Variable region	Constant region	Variable region	Constant region	Variable & Constant region
(-)	13905	100	–				
1	76	1	Emicizumab				
2	14196	102	Negative control	Emicizumab	Negative control	Emicizumab	Negative control
3	3089	22	Emicizumab FIXa arm		Emicizumab FIXa arm		Emicizumab
4	8259	59	Emicizumab FX arm		Emicizumab FX arm		
3+4	57	0	Emicizumab FIXa arm		Emicizumab FIXa arm		
			Emicizumab FX arm		Emicizumab FX arm		
5	12174	88	Negative control		Negative control		
6	13204	95	Emicizumab FIXa arm		Emicizumab FIXa arm		Negative control
7	13062	94	Emicizumab FX arm		Emicizumab FX arm		
8	13621	98	–	Emicizumab Fc region	–	Emicizumab Fc region	–

Note: Relative signal (%) was calculated compared to the ECL signal value of Ab (–) (no antibody treatment) as 100%. ECL signals are shown as means of duplicate measurements.

prolong with the appearance of bleeding events, although APTT's definition of a standardized criterion is difficult due to the inter-reagent, -device, or -laboratory variations of the assay. Additionally, emicizumab concentration was below 30 μg/ml at week 31 before the first bleeding, and then continued to decrease (Figure 2A). Therefore, the measurement of emicizumab concentration is also considered useful for suspecting the appearance of ADAs in patients receiving emicizumab treatment.

In conclusion, our results showed that the appearance of ADAs with neutralizing activity decreased the effect of emicizumab in this patient. Even though our results were obtained from one patient, it was suggested that measuring APTT and emicizumab concentration in plasma may be effective for monitoring a decrease in the efficacy of emicizumab caused by the appearance of ADAs.

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CONFLICT OF INTERESTS

R.K., N.M., Y.T., H.A., Y.I., H.Y., M.S.-N., T.S. and Y.Y. are employees of Chugai Pharmaceutical. T.S. holds patents for inventions relating to anti-FIXa/FX bispecific antibodies, of which all rights have been assigned to the company. R.K., N.M., Y.T., Y.I. and Y.Y. own stock in Chugai Pharmaceutical. M.K. and T.O. receive consulting honoraria from Chugai Pharmaceutical.

AUTHOR CONTRIBUTIONS

Contributions of the individual authors are as follows: Concept: M.S.-N., T.S., Y.Y. and T.O. Design: M.K., R.K., M.S.-N. and T.S. Data acquisition: M.K., R.K., N.M., Y.T., H.A., Y.I. and H.Y. Data interpretation: all. Study supervision: T.S., Y.Y. and T.O. Manuscript drafting: M.K., R.K., N.M.,Y.T., H.A., M.S.-N. and T.S. All authors have reviewed the article and have approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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