

METHOD FOR MEASURING REACTIVITY OF FVIII

Technical Field

5 The present invention relates to methods for measuring the reactivity of FVIII in the presence of a substance having an activity of functionally substituting for coagulation factor VIII (FVIII) (for example, methods for measuring FVIII activity or FVIII inhibitor titer). The present invention also relates to kits and such for measuring the reactivity of FVIII in the presence of a substance having an activity of functionally substituting for FVIII.

Background Art

10 Hemophilia is a hemorrhagic disease caused by a congenital defect or dysfunction of FVIII or coagulation factor IX (FIX). The former is called hemophilia A and the latter is called hemophilia B. Both of these genes are located on the X chromosome; and since they are
15 X-chromosome-linked recessive genetic abnormalities, 99% or more of those who develop the disease are men. It is known that the prevalence rate is approximately one in 10,000 male births, and the ratio between hemophilia A and hemophilia B is approximately 5:1.

 The main bleeding sites in hemophilia patients include intraarticular, intramuscular, subcutaneous, intraoral, intracranial, digestive tract, intranasal, and such. Among them,
20 repeated intraarticular bleeding can develop into hemophilic arthropathy accompanied by articular disorders and difficulty in walking, which eventually may require joint replacement. Therefore, it is a major factor that lowers the QOL of hemophilia patients.

 The severity of hemophilia correlates well with the FVIII activity or FIX activity in blood. Patients with a coagulation factor activity of less than 1% are classified as severe,
25 patients with an activity of 1% or more to less than 5% are classified as moderate, and patients with an activity of 5% or more and less than 40% are classified as mild. Patients with severe symptoms, accounting for approximately half of hemophilia patients, exhibit bleeding symptoms several times a month if they do not receive the later-described preventive replacement therapy, and this frequency is markedly high compared to those of moderately symptomatic patients and
30 mildly symptomatic patients.

 In addition to hemophilia and acquired hemophilia, von Willebrand's disease caused by functional abnormality or deficiency of von Willebrand factor (vWF) is known to be a related bleeding abnormality. vWF is not only necessary for platelets to undergo normal adhesion to the subendothelial tissues at lesion sites of vascular walls, but it is also necessary for forming a
35 complex with FVIII and keeping FVIII in the blood at a normal level. In von Willebrand's disease patients, these functions are decreased, leading to hemostasis dysfunction.

For prevention and/or treatment of bleeding in hemophilia patients, blood coagulation factors purified from plasma or those produced by genetic engineering techniques are mainly used. In severe hemophilia patients, maintaining the FVIII activity or FIX activity in the blood at 1% or more by FVIII or FIX replacement therapy are considered to be effective for preventing manifestation of bleeding symptoms (Non-patent Documents 1 and 2). On the other hand, in hemophilia patients, particularly severe hemophilia patients, antibodies against FVIII or FIX which are called inhibitors may be generated. When such inhibitors are generated, the effect of the coagulation factor formulation is blocked by the inhibitors. As a result, neutralization treatment using large amounts of the coagulation factor formulation, or bypass treatment using a complex concentrate or an activated coagulation factor VII formulation (FVIIa formulation) is carried out.

Measurement of the FVIII activity in hemophilia A is carried out mainly by one-stage clotting assay based on activated partial thromboplastin time (APTT) (Non-patent Document 3) and chromogenic assay which is a system reconstructed using a purified coagulation factor (Non-patent Document 4).

Measurement of the FVIII inhibitor titer in hemophilia A is carried out mainly by Bethesda assay or Nijmegen Bethesda assay (Non-patent Documents 5 and 6).

Recently, a bispecific antibody that binds to both FIX and/or activated coagulation factor IX (FIXa) and coagulation factor X (FX) and/or activated blood coagulation factor X (FXa), and substitutes for the cofactor function of FVIII or more specifically, the function of promoting FX activation by FIXa, was found (Non-patent Documents 7 and 8; Patent Documents 1, 2, and 3). The bispecific antibody functionally substitutes for FVIII to improve the decrease in coagulation reaction due to FVIII deficiency or functional abnormality. For example, with respect to thrombin production and APTT which are indicators of the coagulation reaction, the bispecific antibody shortens the APTT of plasma derived from a hemophilia A patient regardless of the presence of an FVIII inhibitor, and increases the production of thrombin. The APTT-shortening effect of the bispecific antibody was remarkable in comparison to FVIII. This is because FVIII in plasma shows cofactor activity only after activation by activated factor X (FXa) or thrombin, whereas the above-mentioned bispecific antibody does not need such activation process, and for that reason, exhibits the cofactor function more quickly.

Furthermore, antibodies against FIXa Fab and against FX Fab of the bispecific antibody were obtained, and the concentrations of the bispecific antibody in plasma samples from animal testing were determined (Non-patent Document 9).

The bispecific antibody substitutes for the cofactor function of FVIII, thus affecting the assay system that measures the reactivity of FVIII itself. For example, when measuring the plasma FVIII activity by APTT-based one-stage clotting assay to diagnose the severity of

hemophilia A or to monitor the pharmacological activity of an FVIII formulation in an FVIII formulation-administered patient, the action of promoting the shortening of coagulation time of the bispecific antibody strongly interferes in the presence of the bispecific antibody, which greatly impairs the accuracy of measurement. Furthermore, when determining the plasma FVIII inhibitor titer by APTT-based Bethesda assay, the action of promoting the shortening of coagulation time of the bispecific antibody strongly interferes in the presence of the bispecific antibody, which greatly impairs the accuracy of measurement. That is, in patients administered with the bispecific antibody, the FVIII activity and FVIII inhibitor titer cannot be accurately measured. Therefore, methods that enable measurement of the FVIII activity and FVIII inhibitor titer even in the presence of a bispecific antibody are desired.

Citation List

[Non-patent Documents]

- Non-patent Document 1: N Engl J Med. 2007; 357(6): 535-44
- 15 Non-patent Document 2: Thromb Res. 2011; 127 (suppl1):S14-7
- Non-patent Document 3: Thromb Diath Haemorrh. 1962 May 15; 7: 215-28
- Non-patent Document 4: Haemostasis. 1989 19: 196-204.
- Non-patent Document 5: Thromb Diath Haemorrh. 1975; 34(3): 869-72
- Non-patent Document 6: Thromb Haemost. 1995 Feb; 73(2): 247-51.
- 20 Non-patent Document 7: Nat Med. 2012; 18(10): 1570-74
- Non-patent Document 8: PLoS One. 2013; 8(2): e57479.
- Non-patent Document 9: J Thromb Haemost. 2014; 12(2): 206-13 Supporting Information

[Patent Documents]

- Patent Document 1: WO2005/035756
- 25 Patent Document 2: WO2006/109592
- Patent Document 3: WO2012/067176

[Summary of the Invention]

[Problems to be Solved by the Invention]

- 30 The present invention relates to methods for measuring the reactivity of FVIII in the presence of a substance having an activity of functionally substituting for FVIII, for example, methods for measuring FVIII activity or FVIII inhibitor titer. Furthermore, an objective of the present invention is to provide kits or the like for measuring the reactivity of FVIII, such as FVIII activity and FVIII inhibitor titer, in the presence of a substance having an activity of
- 35 functionally substituting for FVIII.

[Means for Solving the Problems]

To solve the above-mentioned problems, the present inventors produced substances that neutralize the activity of the bispecific antibody and by targeting the test items that measure the reactivity of FVIII, searched for measurement conditions that ensure accuracy even in the presence of the bispecific antibody. As a result, the present inventors found out that by using neutralizing antibodies against the bispecific antibody at appropriate concentrations (for example, concentrations at which the bispecific antibody can be sufficiently neutralized), the FVIII activity in the plasma of hemophilia A patients can be evaluated accurately by APTT-based one-stage clotting assay, and also found out that the FVIII inhibitor titer in the plasma of a hemophilia A patient carrying the FVIII inhibitor can be evaluated accurately by APTT-based Bethesda assay. Furthermore, the present inventors successfully discovered kits containing neutralizing antibodies against the bispecific antibody having an FVIII-substituting activity for use in the measurement. The present invention is based on these findings and provides the following:

[1] a method for measuring reactivity of coagulation factor VIII, wherein the method comprises the step of contacting

(1) a blood-derived sample containing a substance that has an activity of functionally substituting for coagulation factor VIII, with

(2) one or more substances that neutralize the substance having an activity of functionally substituting for coagulation factor VIII;

[2] the method of [1], wherein the substance having an activity of functionally substituting for coagulation factor VIII is a bispecific antibody that binds to coagulation factor IX and/or activated coagulation factor IX and to coagulation factor X and/or activated blood coagulation factor X;

[3] the method of [1] or [2], wherein the bispecific antibody is any one of the antibodies described below, in which a first polypeptide is associated with a third polypeptide and a second polypeptide is associated with a fourth polypeptide:

a bispecific antibody in which the first polypeptide is an H chain consisting of the amino acid sequence of SEQ ID NO: 9, the second polypeptide is an H chain consisting of the amino acid sequence of SEQ ID NO: 11, and the third polypeptide and the fourth polypeptide are common L chains of SEQ ID NO: 10 (Q499-z121/J327-z119/L404-k); or

a bispecific antibody in which the first polypeptide is an H chain consisting of the amino acid sequence of SEQ ID NO: 36, the second polypeptide is an H chain consisting of the amino acid sequence of SEQ ID NO: 37, and the third polypeptide and the fourth polypeptide are common L chains of SEQ ID NO: 38 (Q153-G4k/J142-G4h/L180-k);

[4] the method of any one of [1] to [3], wherein the neutralizing substance is one or more

substances selected from the group consisting of peptides, polypeptides, organic compounds, aptamers, and antibodies that neutralize the substance having an activity of functionally substituting for coagulation factor VIII;

[5] the method of any one of [2] to [4], wherein the neutralizing substance is one or more antibodies selected from the group consisting of an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX, an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX, an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X, an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor X, and a bispecific antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX and/or activated coagulation factor IX and Fab comprising an antigen-binding site that binds to coagulation factor X and/or activated coagulation factor X;

[6] the method of any one of [1] to [5], wherein the neutralizing substance is one or more combinations selected from the group consisting of the following antibody combinations:

(a) an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX and an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X;

(b) an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX and an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X;

(c) an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX and an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX; and

(d) an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX, an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X, and an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX;

[7] the method of any one of [1] to [6], wherein the method for measuring reactivity of coagulation factor VIII is a method for measuring the coagulation factor VIII activity or a method for measuring the coagulation factor VIII inhibitor titer;

[8] a kit for use in the method of any one of [1] to [7], wherein the kit comprises one or more antibodies selected from the group consisting of an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX, an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX, an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X, an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor X, and a

bispecific antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX and/or activated coagulation factor IX and Fab comprising an antigen-binding site that binds to coagulation factor X and/or activated coagulation factor X;

[9] the kit of [8], wherein the kit comprises one or more combinations selected from the group consisting of the following antibody combinations:

(a) an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX and an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X;

(b) an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX and an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X;

(c) an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX and an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX; and

(d) an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX, an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X, and an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX;

[10] a method for diagnosing the disease severity of a patient administered with a substance having an activity of functionally substituting for coagulation factor VIII, wherein the method uses the method of any one of [1] to [7];

[11] a method for diagnosing inhibitor titer in a patient administered with a substance having an activity of functionally substituting for coagulation factor VIII, wherein the method uses the method of any one of [1] to [7];

[12] a method for monitoring pharmacological activity of an FVIII formulation in a patient administered with the FVIII formulation and a substance having an activity of functionally substituting for coagulation factor VIII, wherein the method uses the method of any one of [1] to [7];

[13] the method of any one of [10] to [12], wherein the patient is a patient selected from the group consisting of a hemophilia A patient, an acquired hemophilia A patient, a von Willebrand disease patient, and a patient with hemophilia A in which an inhibitor against blood coagulation factor VIII and/or activated blood coagulation factor VIII emerges;

[14] the kit of [8] or [9], wherein the kit is for diagnosing the disease severity of a patient administered with a substance having an activity of functionally substituting for coagulation factor VIII;

[15] the kit of [8] or [9], wherein the kit is for diagnosing inhibitor titer in a patient administered

with a substance having an activity of functionally substituting for coagulation factor VIII;
 [16] the kit of [8] or [9], wherein the kit is for monitoring pharmacological activity of an FVIII
 formulation in a patient administered with the FVIII formulation and a substance having an
 activity of functionally substituting for coagulation factor VIII; and

5 [17] the kit of any one of [14] to [16], wherein the patient is a patient selected from the group
 consisting of a hemophilia A patient, an acquired hemophilia A patient, a von Willebrand disease
 patient, and with a patient with hemophilia A in which an inhibitor against blood coagulation
 factor VIII and/or activated blood coagulation factor VIII emerges.

10 [Effects of the Invention]

The present invention provides methods that can measure FVIII activity and FVIII
 inhibitor titer without being influenced by the activity of a substance having an
 FVIII-substituting activity. A substance having an FVIII-substituting activity includes a
 bispecific antibody that binds to FIX and/or FIXa and FX and/or FXa.

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Brief Description of the Drawings

Fig. 1 shows the results of one-stage clotting assay performed under neutralization of
 the anti-FIXa/FX bispecific antibody using rAQ8-mIgG2b and rAJ540-rbtIgG. When
 FVIII-deficient plasma containing 10 U/dL or 100 U/dL recombinant FVIII supplemented with
 20 an anti-FIXa/FX bispecific antibody, ACE910, was diluted with a buffer (#3 and #7), the FVIII
 activities were shown to be above the range of the calibration curve. On the other hand, when
 FVIII-deficient plasma containing 10 U/dL or 100 U/dL recombinant FVIII supplemented with
 the anti-FIXa/FX bispecific antibody ACE910 was diluted with a buffer containing the two types
 of antibodies against the anti-FIXa/FX bispecific antibody (#4 and #8), the FVIII activities were
 25 shown to be similar to those of the groups without addition of the anti-FIXa/FX bispecific
 antibody (#1 and #5). When FVIII-deficient plasma containing 10 U/dL or 100 U/dL
 recombinant FVIII was diluted with a buffer containing only the two types of antibodies against
 the anti-FIXa/FX bispecific antibody (#2 and #6), the FVIII activities were shown to be similar
 to those of the groups without addition of the anti-FIXa/FX bispecific antibody (#1 and #5).

30 Fig. 2 shows the results of one-stage clotting assay performed under neutralization of
 the anti-FIXa/FX bispecific antibody with AQ1 and AJ541 or AQ1 and AJ522. When
 FVIII-deficient plasma containing 10 U/dL recombinant FVIII supplemented with an
 anti-FIXa/FX bispecific antibody, ACE910, was diluted with a buffer (#4), the FVIII activities
 were shown to be above the range of the calibration curve. On the other hand, when
 35 FVIII-deficient plasma containing 10 U/dL recombinant FVIII supplemented with the
 anti-FIXa/FX bispecific antibody ACE910 was diluted with a buffer containing two types of

antibodies, AQ1 and AJ541, against the anti-FIXa/FX bispecific antibody (#5), or a buffer containing two types of antibodies, AQ1 and AJ522, against the anti-FIXa/FX bispecific antibody (#6), the FVIII activities were shown to be similar to those of the groups without addition of the anti-FIXa/FX bispecific antibody (#1). When FVIII-deficient plasma containing 10 U/dL recombinant FVIII was diluted with a buffer containing only the two types of antibodies, AQ1 and AJ541, against the anti-FIXa/FX bispecific antibody (#2), or a buffer containing only the two types of antibodies, AQ1 and AJ522 (#3), the FVIII activities were shown to be similar to those of the groups without addition of the anti-FIXa/FX bispecific antibody (#1).

Fig. 3 shows the results of one-stage clotting assay performed under neutralization of the anti-FIXa/FX bispecific antibody with AQ512 and AJ114 or AQ512 and AJ521. When FVIII-deficient plasma containing 10 U/dL recombinant FVIII supplemented with an anti-FIXa/FX bispecific antibody, hBS23, was diluted with a buffer (#4), the FVIII activities were shown to be above the range of the calibration curve. On the other hand, when FVIII-deficient plasma containing 10 U/dL recombinant FVIII supplemented with the anti-FIXa/FX bispecific antibody hBS23 was diluted with a buffer containing two types of antibodies, AQ512 and AJ114, against the anti-FIXa/FX bispecific antibody (#5), or a buffer containing two types of antibodies, AQ512 and AJ521, against the anti-FIXa/FX bispecific antibody (#6), the FVIII activities were shown to be similar to those of the groups without addition of the anti-FIXa/FX bispecific antibody (#1). When FVIII-deficient plasma containing 10 U/dL recombinant FVIII was diluted with a buffer containing only the two types of antibodies, AQ512 and AJ114, against the anti-FIXa/FX bispecific antibody (#2), or a buffer containing only the two types of antibodies, AQ512 and AJ521 (#3), the FVIII activities were shown to be similar to those of the groups without addition of the anti-FIXa/FX bispecific antibody (#1).

Fig. 4 shows the results of Bethesda assay performed under neutralization of the anti-FIXa/FX bispecific antibody using rAQ8-mIgG2b and rAJ540-rbtIgG. FVIII inhibitor plasma containing only the anti-FIXa/FX bispecific antibody ACE910 (#3) showed an activity equivalent to 100% or more of FVIII of the calibration curve. On the other hand, FVIII inhibitor plasma containing the anti-FIXa/FX bispecific antibody and the two types of antibodies against the anti-FIXa/FX bispecific antibody (#4) showed an FVIII inhibitor titer similar to that of the inhibitor plasma without additives (#1). FVIII inhibitor plasma containing only the two types of antibodies against the anti-FIXa/FX bispecific antibody (#2) showed results similar to that of #1.

Mode for Carrying Out the Invention

The method for measuring FVIII activity of the present invention comprises the step of contacting (1) and (2) described below. Otherwise, the method can be carried out according to

methods generally used for measuring FVIII activity. Details will be explained in the Examples as well.

(1) a blood-derived sample containing a substance having an activity of functionally substituting for FVIII

5 (2) a substance that neutralizes the substance having an activity of functionally substituting for FVIII

Methods for measuring FVIII activity

10 The FVIII activity measurement methods that are generally used and known to those skilled in the art can be used, and for example, one can use a one-stage clotting assay (Casillas *et al.*, (1971) Coagulation 4: 107-11) that uses factor VIII-deficient plasma (Sysmex, Kobe, Japan), which is based on coagulation time (aPTT measurements). One-stage clotting assay is carried out, for example, by the following method. Three solutions, 50 μ L of ten-fold diluted test plasma, 50 μ L of FVIII-deficient plasma, and 50 μ L of an APTT reagent are mixed; and this is
15 incubated at 37°C for five minutes, followed by addition of 50 μ L of a calcium solution to initiate the coagulation reaction, and then the time to coagulation is measured. Furthermore, instead of the test plasma, serially diluted samples of normal plasma (FVIII activity in a ten-fold diluted normal plasma is specified as 100%) are measured, and a calibration curve is produced by plotting the FVIII activity on the horizontal axis and coagulation time on the vertical axis.
20 The coagulation time of the test plasma is converted to FVIII activity using the calibration curve, and FVIII activity in the test plasma is calculated. Herein, unless stated otherwise, the phrase “measurement of FVIII activity” is used as a phrase that may include “measurement of activated coagulation factor VIII (FVIIIa) activity”.

25 In addition to one-stage clotting assay, thrombin generation assay (TGA), measurement methods that use rotation thromboelastometry, FVIII chromogenic assay, coagulation waveform analysis, thrombin and activated factor X production assay, and such may be used as the method for measuring FVIII activity. The method for measuring FVIII inhibitor titer of the present invention includes the step of contacting (1) and (2) described below. Otherwise, the method can be carried out according to generally used methods for measuring FVIII inhibitor titer.

30 Details will be explained in the Examples as well.

(1) a blood-derived sample containing a substance having an activity of functionally substituting for FVIII

(2) a substance that neutralizes the substance having an activity of functionally substituting for FVIII

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Methods for measuring FVIII inhibitor titer

The FVIII inhibitor titer measurement methods that are generally used and known to those skilled in the art can be used, and for example, one can use Bethesda assay (Kasper *et al.*, (1975) *Thrombos Diath Haemorrh* 34: 869-872), ELISA method, and Nijmegen Bethesda assay (Nijmegen modification assay) (Verbruggen *et al.*, (1995) *Thromb Haemost* 73: 247-251).

5 Bethesda assay is carried out, for example, by the following method. A solution produced by mixing equal amounts of normal plasma and test plasma is incubated at 37°C for two hours, and then the residual factor VIII activity in normal plasma is measured by one-stage clotting assay based on activated partial thromboplastin time (APTT). The action of inhibiting 50% of the factor VIII activity in normal plasma is specified as 1 Bethesda (1BU), and therefore the FVIII
10 inhibitor titer is calculated in units of Bethesda. When the FVIII inhibitor titer in the test plasma is high and the residual FVIII activity does not lie within the range of 25% to 75%, test plasma suitably diluted with a buffer is used to recalculate the Bethesda units, and subsequently, the value is multiplied by the dilution ratio to calculate the FVIII inhibitor titer in the test plasma.

15 FVIII

FVIII is one of a series of molecules involved in blood coagulation, which demonstrates cofactor activity when it is activated by thrombin or FIXa and promotes the FX activation reaction by FIXa.

20 FVIII inhibitor

The FVIII inhibitor is an isoantibody against foreign FVIII and is emerged in 20% to 30% of hemophilia A patients. An individual who is originally normal may produce autoantibodies against FVIII posteriori. Generally, most FVIII inhibitor isoantibodies and autoantibodies function as anti-FVIII neutralizing antibodies, and decrease or eliminate FVIII
25 activity.

Activity of substituting for FVIII

A substance having an activity of functionally substituting for FVIII of the present invention can be rephrased as a substance having an FVIII-like activity. In the present
30 invention, the phrase “functionally substitute/substituting for FVIII” means that FX activation by FIXa is promoted (FXa generation by FIXa is promoted). More specifically, in the present invention, the phrase “functionally substitute/substituting for FVIII” means recognizing FIX and/or FIXa, and FX and/or FXa, and promoting activation of FX by FIXa (promoting FXa generation by FIXa). The activity of promoting FXa generation can be evaluated using, for
35 example, a measurement system comprising FIXa, FX, synthetic substrate S-2222 (synthetic substrate of FXa), and phospholipids. Such measurement system shows correlation between

the severity of the disease and clinical symptoms in hemophilia A cases (Rosen S, Andersson M, Blomback M *et al.* Clinical applications of a chromogenic substrate method for determination of FVIII activity. *Thromb Haemost* 1985; 54: 811-23).

A preferred embodiment of a substance having an activity of functionally substituting for FVIII of the present invention includes, for example, a bispecific antibody that binds to FIX and/or FIXa, and to FX and/or FXa. Such an antibody can be obtained according to methods described, for example, in WO2005/035756, WO2006/109592, and WO2012/067176. The bispecific antibody of the present invention includes antibodies described in these documents.

A preferred bispecific antibody includes, for example, ACE910 (Q499-z121/J327-z119/L404-k) (a bispecific antibody in which the H chain consisting of the amino acid sequence of SEQ ID NO: 9 and the L chain of SEQ ID NO: 10 are associated, and the H chain consisting of the amino acid sequence of SEQ ID NO: 11 and the L chain of SEQ ID NO: 10 are associated) and hBS23 (Q153-G4k/J142-G4h/L180-k) (a bispecific antibody in which the H chain consisting of the amino acid sequence of SEQ ID NO: 36 and the L chain of SEQ ID NO: 38 are associated, and the H chain consisting of the amino acid sequence of SEQ ID NO: 37 and the L chain of SEQ ID NO: 38 are associated), which are bispecific antibodies described in a patent document (WO2012/067176).

Neutralization

“Neutralization” in a substance that neutralizes the substance having an activity of functionally substituting for FVIII in the present invention refers to, for example, complete or partial inhibition of the activity of functionally substituting for FVIII of a substance that has an activity of functionally substituting for FVIII. For example, when the substance having the activity of functionally substituting for FVIII is an antibody, complete or partial inhibition of the activity of functionally substituting for FVIII may be accomplished by completely or partially inhibiting binding of the antibody to the antigen, but is not limited thereto

Neutralizing substances

The term “substance” of the neutralizing substance in the substance that neutralizes the substance having an activity of functionally substituting for FVIII in the present invention refers to, for example, peptides, polypeptides, organic compounds, aptamers, antibodies, and such that bind to the substance having an activity of functionally substituting for FVIII.

A plurality of neutralizing substances can be used in combination, and for example, antibodies and aptamers can be used in combination.

Polypeptides

Polypeptides in the present invention normally refer to proteins and peptides having a length of approximately ten amino acids or longer. Generally, they are biologically derived polypeptides, but are not particularly limited to such polypeptides, and may be, for example, polypeptides comprising an artificially designed sequence. Furthermore, they may be any native polypeptides, or synthetic polypeptides, recombinant polypeptides, or such. Additionally, the fragments of the above-mentioned polypeptide are also included in the polypeptides of the present invention.

Organic compounds

Organic compounds in the present invention are, for example, low-molecular-weight compounds, preferably with a molecular weight of 1000 or less.

Aptamers

The term "aptamer" refers to a nucleic acid molecule that binds specifically to a target molecule such as a polypeptide. For example, aptamers of the present invention can be RNA aptamers capable of binding specifically to substances having an FVIII-substituting activity. Production and therapeutic use of aptamers are well established in this field. For example, aptamers can be obtained by using the SELEX method (see U.S. Patent Nos. 5475096, 5580737, 5567588, 5707796, 5763177, 6699843, and such).

Antibodies

When the substance having an activity of functionally substituting for FVIII is a bispecific antibody that binds to FIX and/or FIXa and to FX and/or FXa, examples of antibodies that bind to the substance having an activity of functionally substituting for FVIII include antibodies selected from the group consisting of antibodies that bind to Fab containing an antigen-binding site that binds to FIX, antibodies that bind to Fab containing an antigen-binding site that binds to FIXa, antibodies that bind to Fab containing an antigen-binding site that binds to FX, antibodies that bind to Fab containing an antigen-binding site that binds to FXa, and bispecific antibodies that bind to Fab containing an antigen-binding site that binds to FIX and/or FIXa and to Fab containing an antigen-binding site that binds to FX and/or FXa. The above-mentioned antibodies can be used separately or in multi-combinations. For example, it is possible to use multiple antibodies that bind to Fab containing an antigen-binding site that binds to one type of antigen, for example, multiple types of antibodies that bind to Fab containing an antigen-binding site that binds to FIX. For example, when the substance having an activity of functionally substituting for FVIII is a bispecific antibody that binds to FIX and/or FIXa and to FX and/or FXa, the following antibody combinations can be used:

(a) an antibody that binds to Fab containing an antigen-binding site that binds to FIX and an antibody that binds to Fab containing an antigen-binding site that binds to FX;

(b) an antibody that binds to Fab containing an antigen-binding site that binds to FIXa and an antibody that binds to Fab containing an antigen-binding site that binds to FX;

5 (c) an antibody that binds to Fab containing an antigen-binding site that binds to FIX and an antibody that binds to Fab containing an antigen-binding site that binds to FIXa; and

(d) an antibody that binds to Fab containing an antigen-binding site that binds to FIX, an antibody that binds to Fab containing an antigen-binding site that binds to FX, and an antibody that binds to Fab containing an antigen-binding site that binds to FIXa.

10 An example of an antibody that binds to Fab containing an antigen-binding site that binds to FIX and/or FIXa includes the AQ8, AQ1, and AQ512 antibodies. The nucleotide sequences of the variable regions and the amino acid sequences predicted therefrom were analyzed by GENETYX Ver. 9 (GENETYX CORPORATION).

The amino acid sequence and the nucleotide sequence of the H chain variable region of AQ8
15 are indicated by the following SEQ ID NOs:

amino acid sequence: SEQ ID NO: 1; and

nucleotide sequence: SEQ ID NO: 5.

The amino acid sequence and the nucleotide sequence of the L chain variable region of AQ8 are indicated by the following SEQ ID NOs:

20 amino acid sequence: SEQ ID NO: 2; and

nucleotide sequence: SEQ ID NO: 6.

The amino acid sequences and the nucleotide sequences of the H-chain CDRs 1 to 3 of AQ8 are indicated by the following SEQ ID NOs:

CDR1 amino acid sequence: SEQ ID NO: 12;

25 CDR2 amino acid sequence: SEQ ID NO: 13;

CDR3 amino acid sequence: SEQ ID NO: 14;

CDR1 nucleotide sequence: SEQ ID NO: 15;

CDR2 nucleotide sequence: SEQ ID NO: 16; and

CDR3 nucleotide sequence: SEQ ID NO: 17.

30 The amino acid sequences and the nucleotide sequences of the L-chain CDRs 1 to 3 of AQ8 are indicated by the following SEQ ID NOs:

CDR1 amino acid sequence: SEQ ID NO: 18;

CDR2 amino acid sequence: SEQ ID NO: 19;

CDR3 amino acid sequence: SEQ ID NO: 20;

35 CDR1 nucleotide sequence: SEQ ID NO: 21;

CDR2 nucleotide sequence: SEQ ID NO: 22; and

CDR3 nucleotide sequence: SEQ ID NO: 23.

An example of an antibody that binds to Fab containing an antigen-binding site that binds to FX and/or FXa includes the AJ540, AJ541, AJ522, AJ114, and AJ521 antibodies. The nucleotide sequences of the variable regions and the amino acid sequences predicted therefrom were analyzed by GENETYX Ver. 9 (GENETYX CORPORATION).

The amino acid sequence and the nucleotide sequence of the H chain variable region of AJ540 are indicated by the following SEQ ID NOs:

amino acid sequence: SEQ ID NO: 3; and

nucleotide sequence: SEQ ID NO: 7.

The amino acid sequence and the nucleotide sequence of the L chain variable region of AJ540 are indicated by the following SEQ ID NOs:

amino acid sequence: SEQ ID NO: 4; and

nucleotide sequence: SEQ ID NO: 8.

The amino acid sequences and the nucleotide sequences of the H-chain CDRs 1 to 3 of AJ540 are indicated by the following SEQ ID NOs:

CDR1 amino acid sequence: SEQ ID NO: 24;

CDR2 amino acid sequence: SEQ ID NO: 25;

CDR3 amino acid sequence: SEQ ID NO: 26;

CDR1 nucleotide sequence: SEQ ID NO: 27;

CDR2 nucleotide sequence: SEQ ID NO: 28; and

CDR3 nucleotide sequence: SEQ ID NO: 29.

The amino acid sequences and the nucleotide sequences of the L-chain CDRs 1 to 3 of AJ540 are indicated by the following SEQ ID NOs:

CDR1 amino acid sequence: SEQ ID NO: 30;

CDR2 amino acid sequence: SEQ ID NO: 31;

CDR3 amino acid sequence: SEQ ID NO: 32;

CDR1 nucleotide sequence: SEQ ID NO: 33;

CDR2 nucleotide sequence: SEQ ID NO: 34; and

CDR3 nucleotide sequence: SEQ ID NO: 35.

The term “antibody” is used in the broadest sense, and may be monoclonal antibodies, polyclonal antibodies, dimers, multimers, multispecific antibodies (for example, bispecific antibodies), antibody derivatives, and modified antibody products (Miller K *et al.* J Immunol. 2003, 170(9), 4854-61) as long as they display a desired biological activity. The antibodies may be mouse antibodies, human antibodies, humanized antibodies, chimeric antibodies, or those derived from another species, or they may be artificially synthesized antibodies. The antibodies disclosed herein can be of any type (for example, IgG, IgE, IgM, IgD, and IgA), class

(for example, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecules. The immunoglobulins can be derived from any species (for example, human, mouse, or rabbit). The terms “antibody”, “immune globulin” and “immunoglobulin” are used interchangeably in a broad sense.

5 The term “antibody derivative” includes a portion of an antibody, preferably an antibody variable region, or at least an antigen-binding region of an antibody. Antibody derivatives include, for example, Fab, Fab’, F(ab’)₂, Fv fragments, linear antibodies, and single-chain antibodies (scFv), sc(Fv)₂, Fab₃, domain antibodies (dAb) (WO2004/058821, WO2003/002609), diabodies, triabodies, tetrabodies, minibodies, and multispecific antibodies formed from antibody
10 derivatives, but are not limited thereto. Here, “Fab” is constructed from a single light chain and the CH1 domain and variable region of a single heavy chain. Furthermore, “Fv” is the smallest antibody derivative, and includes a complete antigen-recognizing region and an antigen-binding region. The antibody derivative may be, for example, a fusion between an IgG antibody and Fc. For example, one can refer to Example 2 in U.S. Patent No. 5641870 specification; Zapata G *et al.* Protein Eng. 1995, 8(10), 1057-1062; Olafsen T *et al.* Protein Eng. Design & Sel. 2004, 17(4): 315-323; Holliger P *et al.* Nat. Biotechnol. 2005, 23(9): 1126-36; Fischer N *et al.* Pathobiology. 2007, 74(1): 3-14; Shen J *et al.* J Immunol Methods. 2007, 318, 65-74; and Wu *et al.* Nat Biotechnol. 2007, 25(11), 1290-7.

 Examples of modified antibody products may include antibodies linked to various
20 molecules such as polyethylene glycol (PEG). Antibodies of the present invention include such modified antibody products. The substance to be linked is not limited in the modified antibody products of the present invention. To yield such modified antibody products, chemical modifications can be made to the obtained antibodies. Such methods are already established in this field.

25 “Bispecific” antibodies refer to antibodies having variable regions that recognize different epitopes, where the regions are within the same antibody molecule. Bispecific antibodies may be antibodies that recognize two or more different antigens or antibodies that recognize two or more different epitopes on the same antigen. Bispecific antibodies may include not only whole antibodies but antibody derivatives. Antibodies of the present invention
30 also include bispecific antibodies. Herein, anti-FIXa/FX bispecific antibody and bispecific antibody that binds to FIXa and FX are used synonymously.

Methods for producing genetically engineered antibodies

 Recombinant antibodies produced by using genetic engineering techniques can be used
35 as the antibodies. Recombinant antibodies can be obtained by cloning DNAs encoding the antibodies from hybridomas or antibody-producing cells such as sensitized lymphocytes that

produce antibodies, inserting them into vectors, and then introducing them into hosts (host cells) to produce the antibodies.

The antibodies include human antibodies, mouse antibodies, and rat antibodies, and their origin is not limited. They may also be genetically modified antibodies such as chimeric antibodies and humanized antibodies.

Methods for obtaining human antibodies are known. For example, transgenic animals carrying the entire repertoire of human antibody genes can be immunized with antigens of interest to obtain human antibodies of interest (see International Publication WO 93/12227, WO 92/03918, WO 94/02602, WO 94/25585, WO 96/34096, and WO 96/33735).

Genetically modified antibodies can be produced using known methods. Specifically, for example, chimeric antibodies comprise H chain and L chain variable regions of an immunized animal antibody, and H chain and L chain constant regions of a human antibody. Chimeric antibodies can be obtained by linking DNAs encoding the variable regions of the antibody derived from the immunized animal, with DNAs encoding the constant regions of a human antibody, inserting this into an expression vector, and then introducing it into host to produce the antibodies.

Humanized antibodies are modified antibodies that are also referred to as reshaped human antibodies. A humanized antibody is constructed by transferring the CDRs of an antibody derived from an immunized animal to the complementarity determining regions of a human antibody. Conventional genetic recombination techniques for such purposes are known (see European Patent Application Publication No. EP 239400; International Publication No. WO 96/02576; Sato K *et al.*, Cancer Research 1993, 53: 851-856; International Publication No. WO 99/51743).

Bispecific antibodies are antibodies that have specificity to two different antigens.

While bispecific antibodies are not limited to those of the IgG type, for example, IgG-type bispecific antibodies can be secreted from a hybrid hybridoma (quadroma) produced by fusing two types of hybridomas that produce IgG antibodies (Milstein C. *et al.*, Nature 1983, 305: 537-540). They can also be secreted by introducing the L chain and H chain genes constituting the two types of IgGs of interest, a total of four types of genes, into cells to co-express the genes.

In this case, by introducing suitable amino acid substitutions to the CH3 regions of the H chains, IgGs having a heterogeneous combination of H chains can be preferentially secreted (Ridgway JB *et al.* Protein Engineering 1996, 9: 617-621; Merchant AM *et al.* Nature Biotechnology 1998, 16: 677-681; WO 2006/106905; Davis JH *et al.* Protein Eng Des Sel. 2010, 4: 195-202).

Regarding the L chains, since the diversity of L chain variable regions is lower than that

of H chain variable regions, one can expect to obtain common L chain that can confer binding ability to both H chains. The antibodies of the present invention may be antibodies comprising common L chains. Bispecific IgGs can be efficiently expressed by introducing the gene of the common L chain and both H chains into cells.

5

Epitopes

Antibodies which are an embodiment of substances that neutralize the substance having an activity of functionally substituting for FVIII of in present invention include antibodies that bind to an epitope overlapping with an epitope bound by the antibodies described above, and preferably antibodies that bind to the same epitope.

10

Whether an antibody recognizes the same epitope as or an epitope overlapping with an epitope that is recognized by another antibody can be confirmed by competition between the two antibodies against the epitope. Competition between the antibodies can be evaluated by competitive binding assays using means such as enzyme-linked immunosorbent assay (ELISA), fluorescence energy transfer method (FRET), and fluorometric microvolume assay technology (FMAT (Registered trademark)). The amount of antibodies bound to an antigen indirectly correlate with the binding ability of candidate competitor antibodies (test antibodies) that competitively bind to the same or overlapping epitope. In other words, as the amount of or the affinity of test antibodies against the same or overlapping epitope increases, the amount of antibodies bound to the antigen decreases, and the amount of test antibodies bound to the antigen increases. Specifically, the appropriately labeled antibodies and test antibodies are simultaneously added to the antigens, and then the bound antibodies are detected using the label. The amount of the antibodies bound to the antigen can be easily determined by labeling the antibodies in advance. This label is not particularly limited, and the labeling method is selected according to the assay technique used. Specific examples of the labeling method include fluorescent labeling, radiolabeling, and enzyme labeling.

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Herein, the “antibody that binds to the overlapping epitope” or “antibody that binds to the same epitope” refers to a test antibody that can reduce the amount of binding of the labeled antibody by at least 50% at a concentration that is usually 100 times higher, preferably 80 times higher, more preferably 50 times higher, even more preferably 30 times higher, and still more preferably 10 times higher than a concentration of the non-labeled antibody at which binding of the non-labeled antibody reduces the amount of binding of the labeled antibody by 50% (IC_{50}). The epitope recognized by the antibody can be analyzed by methods known to those skilled in the art, and for example, it can be performed by Western blotting and such.

35

Antibody production methods

Antibodies of the present invention can be produced by methods known to those skilled in the art. Specifically, DNA encoding the antibody of interest is inserted into an expression vector. Insertion into an expression vector is carried out such that the expression will take place under the control of expression regulatory regions such as enhancers and promoters. Next, host cells are transformed using this expression vector to express the antibodies. Appropriate combinations of the host and expression vector can be used in this step.

Examples of the vectors include M13 series vectors, pUC series vectors, pBR322, pBluescript, and pCR-Script. In addition to these vectors, for example, pGEM-T, pDIRECT, or pT7 can also be used for the purpose of cDNA subcloning and excision.

Particularly, expression vectors are useful for using the vectors for the purpose of producing the antibody. For example, when the host is *E. coli* such as JM109, DH5 α , HB101, or XL1-Blue, the expression vectors indispensably have a promoter that permits efficient expression in *E. coli*, for example, lacZ promoter (Ward *et al.*, Nature (1989) 341, 544-546; and FASEB J (1992) 6, 2422-2427), araB promoter (Better *et al.*, Science (1988) 240, 1041-1043), or T7 promoter. Examples of such vectors include the vectors mentioned above as well as pGEX-5X-1 (manufactured by Pharmacia), "QIAexpress system" (manufactured by QIAGEN), pEGFP, and pET (in this case, the host is preferably BL21 expressing T7 RNA polymerase).

The vectors may contain a signal sequence for polypeptide secretion. In the case of production in the periplasm of *E. coli*, pelB signal sequence (Lei, S. P. *et al.*, J. Bacteriol. (1987) 169, 4397) can be used as the signal sequence for polypeptide secretion. The vectors can be transferred to the host cells using, for example, calcium chloride methods or electroporation methods.

In addition to the *E. coli* expression vectors, examples of the vectors for producing the antibody of the present invention include mammal-derived expression vectors (*e.g.*, pcDNA3 (manufactured by Invitrogen Corp.), pEGF-BOS (Nucleic Acids. Res. 1990, 18(17), p5322), pEF, and pCDM8), insect cell-derived expression vectors (*e.g.*, "Bac-to-BAC baculovirus expression system" (manufactured by GIBCO BRL), and pBacPAK8), plant-derived expression vectors (*e.g.*, pMH1 and pMH2), animal virus-derived expression vectors (*e.g.*, pHSV, pMV, and pAdexLcw), retrovirus-derived expression vectors (*e.g.*, pZIPneo), yeast-derived expression vectors (*e.g.*, "Pichia Expression Kit" (manufactured by Invitrogen Corp.), pNV11, and SP-Q01), and *Bacillus subtilis*-derived expression vectors (*e.g.*, pPL608 and pKTH50).

For the purpose of expression in animal cells such as CHO cells, COS cells, or NIH3T3 cells, the vectors indispensably have a promoter necessary for intracellular expression, for example, SV40 promoter (Mulligan *et al.*, Nature (1979) 277, 108), MMTV-LTR promoter, EF1 α promoter (Mizushima *et al.*, Nucleic Acids Res (1990) 18, 5322), CAG promoter (Gene (1991) 108, 193), or CMV promoter and, more preferably, have a gene for screening for

transformed cells (*e.g.*, a drug resistance gene that can work as a marker by a drug (neomycin, G418, *etc.*)). Examples of the vectors having such properties include pMAM, pDR2, pBK-RSV, pBK-CMV, pOPRSV, and pOP13.

An exemplary method intended to stably express the gene and increase the number of intracellular gene copies involves transfecting CHO cells deficient in nucleic acid synthesis pathway with vectors having a DHFR gene serving as a complement thereto (*e.g.*, pCHOI) and using methotrexate (MTX) in the gene amplification. An exemplary method intended to transiently express the gene involves using COS cells having a gene which expresses an SV40 T antigen on their chromosomes to transform the cells with vectors having a replication origin of SV40 (pcD, *etc.*). Also, a replication origin derived from polyomavirus, adenovirus, bovine papillomavirus (BPV), or the like may be used. The expression vectors for increasing the number of gene copies in a host cell system can additionally contain a selection marker such as an aminoglycoside transferase (APH) gene, a thymidine kinase (TK) gene, an *E. coli* xanthine guanine phosphoribosyltransferase (Ecogpt) gene, or a dihydrofolate reductase (dhfr) gene.

The antibodies of the present invention obtained by the methods described above can be isolated from inside host cells or from outside of the cells (the medium, or such), and purified to practically pure and homogeneous antibodies. The antibodies can be separated and purified by methods routinely used for separating and purifying antibodies, and the type of method is not limited. For example, the antibodies can be separated and purified by appropriately selecting and combining column chromatography, filtration, ultrafiltration, salting-out, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectrofocusing, dialysis, recrystallization, and such.

The chromatographies include, for example, affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel filtration, reverse phase chromatography, and adsorption chromatography (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak *et al.*, Cold Spring Harbor Laboratory Press, 1996). The chromatographic methods described above can be conducted using liquid-chromatography, for example, HPLC and FPLC. Columns used for affinity chromatography include protein A columns and protein G columns. Columns using protein A include, for example, Hyper D, POROS, and Sepharose FF (GE Amersham Biosciences). The present invention includes antibodies that are highly purified using these purification methods.

The obtained antibodies can be purified to homogeneity. Separation and purification of the antibodies can be performed using separation and purification methods generally used for protein separation and purification. For example, the antibodies can be separated and purified by appropriately selecting and combining column chromatography such as affinity chromatography, filtration, ultrafiltration, salting-out, dialysis, SDS-polyacrylamide gel

electrophoresis, isoelectric focusing, and such, without limitation (Antibodies: A Laboratory Manual. Ed Harlow and David Lane, Cold Spring Harbor Laboratory, 1988). Columns used for affinity chromatography include, for example, protein A columns and protein G columns.

5 Methods for obtaining samples

In the present invention, blood-derived samples are preferably blood-derived samples collected from a test subject. Such blood-derived samples can be obtained from test subjects administered with a substance having an FVIII-substituting activity. A test subject includes, for example, a patient with hemorrhagic symptoms at any part in the body (hemorrhagic disease patient). The main bleeding sites are intraarticular, intramuscular, subcutaneous, intraoral, intracranial, digestive tract, intranasal, and such, but are not limited thereto. The hemorrhagic disease patient is preferably a patient with hemorrhagic disease caused by decrease or deficiency in an FVIII activity and/or FVIIIa activity. The patient with hemorrhagic disease caused by decrease or deficiency in the FVIII activity and/or FVIIIa activity is a patient with hemorrhagic symptoms, and examples include patients with a priori or posteriori decrease or deficiency in either or both of the FVIII activity and FVIIIa activity. Decrease in the activities of FVIII and FVIIIa means that in comparison to those of healthy individuals, these activities are preferably less than 40% (for example, less than 40%, less than 30%, less than 20%, or less than 10%), more preferably less than 10% (for example, less than 10%, less than 9%, less than 8%, less than 7%, or less than 6%), even more preferably less than 5% (for example, less than 5%, less than 4%, less than 3%, or less than 2%), and particularly preferably less than 1% in a patient, without being limited thereto.

More specifically, examples of such diseases include diseases selected from among hemophilia (hemophilia A and hemophilia B), acquired hemophilia, and von Willebrand's disease caused by functional abnormality or deficiency of von Willebrand factor (vWF), but are not limited thereto. Blood-derived samples include serum, plasma, or whole blood. In the present invention, use of plasma samples is preferred. Methods for obtaining blood-derived samples from test subjects are well known to those skilled in the art.

30 Kits

Various types of reagents such as buffers required for the method for measuring the reactivity of FVIII of the present invention can be packaged in advance and provided as a kit. The kit of the present invention may include in addition to the buffer, plasma samples isolated from a human whose FVIII activity and FIX activity in the blood are normal, a substance having an FVIII-substituting activity, and anything that can be used in FVIII activity measurement, or anything that can be used in FVIII inhibitor titer measurement. Furthermore, the various types

of reagents included in the kit can be made into a powder or liquid form according to their mode of use. Furthermore, they can be stored in appropriate containers and used when suitable.

The disease severity of a patient administered with the substance having an activity of functionally substituting for FVIII, for example, can be diagnosed by using the method of the present invention. Reactivity of FVIII can be measured using the method of this invention, and the disease severity and/or inhibitor titer for the patient can be diagnosed/assessed based on the measurement results. The diagnosis and assessment methods can be performed by methods known to those skilled in the art.

The pharmacological activity of an FVIII formulation in patients administered with the FVIII formulation and a substance having an activity of functionally substituting for FVIII, for example, can be monitored by using the methods of the present invention. Monitoring can be carried out by methods known to those skilled in the art.

The kit of the present invention can be used as a kit for diagnosing the disease severity of a patient administered with a substance having an activity of functionally substituting for FVIII. Reactivity of FVIII can be measured using the kit of this invention, and the disease severity of the patient can be diagnosed/assessed based on the measurement results. The diagnosis and assessment methods can be performed by methods known to those skilled in the art.

The kit of the present invention can be used, for example, as a kit for monitoring the pharmacological activity of an FVIII formulation in a patient administered with the FVIII formulation and a substance having an activity of functionally substituting for FVIII. Monitoring can be carried out by methods known to those skilled in the art.

For example, one may use a method for treating a patient, which comprises the steps of:

- (a) administering a first dose of a substance having an activity of functionally substituting for FVIII;
- (b) monitoring the reactivity of FVIII in the patient;
- (c) determining a second dose of the substance having an activity of functionally substituting for FVIII based on the observed reactivity of FVIII; and
- (d) administering to the patient the second dose of the substance having an activity of functionally substituting for FVIII.

Furthermore, one may use, for example, a method for treating a patient, which comprises the steps of:

- (a) administering a substance having an activity of functionally substituting for FVIII following a first administration interval;
- (b) monitoring the reactivity of FVIII in the patient;
- (c) determining a second administration interval for the substance having an activity of

functionally substituting for FVIII based on the observed reactivity of FVIII; and

(d) administering to the patient the substance having an activity of functionally substituting for FVIII following the second administration interval.

One may also use, for example, a method for treating a patient, which comprises
 5 monitoring the reactivity of FVIII, and changing the administration dose and/or the
 administration interval of the substance having an activity of functionally substituting for
 coagulation factor VIII depending on the reactivity of FVIII.

The substance having an activity of functionally substituting for FVIII is preferably a
 bispecific antibody that binds to FIX and/or FIXa and to FX and/or FXa. It is more preferably
 10 the antibody described below, which is a bispecific antibody in which a first polypeptide is
 associated with a third polypeptide and a second polypeptide is associated with a fourth
 polypeptide

bispecific antibody in which the first polypeptide is an H chain consisting of the amino acid
 sequence of SEQ ID NO: 9, the second polypeptide is an H chain consisting of the amino acid
 15 sequence of SEQ ID NO: 11, and the third polypeptide and the fourth polypeptide are common L
 chains of SEQ ID NO: 10 (Q499-z121/J327-z119/L404-k), or

bispecific antibody in which the first polypeptide is an H chain consisting of the amino acid
 sequence of SEQ ID NO: 36, the second polypeptide is an H chain consisting of the amino acid
 sequence of SEQ ID NO: 37, and the third polypeptide and the fourth polypeptide are common L
 20 chains of SEQ ID NO: 38 (Q153-G4k/J142-G4h/L180-k).

The dose is, for example, 0.001 mg/kg to 100 mg/kg for the aforementioned bispecific
 antibody. It is preferably approximately 0.001 mg/kg, approximately 0.003 mg/kg,
 approximately 0.005 mg/kg, approximately 0.01 mg/kg, approximately 0.03 mg/kg,
 approximately 0.05 mg/kg, approximately 0.1 mg/kg, approximately 0.3 mg/kg, approximately
 25 0.5 mg/kg, approximately 1 mg/kg, approximately 3 mg/kg, approximately 5 mg/kg,
 approximately 10 mg/kg, approximately 20 mg/kg, approximately 30 mg/kg, approximately 40
 mg/kg, approximately 50 mg/kg, approximately 60 mg/kg, approximately 70 mg/kg,
 approximately 80 mg/kg, approximately 90 mg/kg, and approximately 100 mg/kg. The doses
 before and after the monitoring step may be the same or different. In the case of the
 30 aforementioned bispecific antibody, the administration interval is, for example, at least one day
 or more. The interval is preferably 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2
 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12
 weeks, 13 weeks, 14 weeks, 15 weeks, 16 weeks, 17 weeks, 18 weeks, 19 weeks, 20 weeks, 21
 weeks, 22 weeks, 23 weeks, 24 weeks, 25 weeks, 1 month, 2 months, 3 months, 4 months, 5
 35 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, or 1 year. The dose
 intervals before and after the monitoring step may be the same or different.

The target patients for the methods or kits of the present invention are, for example, hemophilia A patients, acquired hemophilia A patients, von Willebrand disease patients, and hemophilia A patients with emergence of an inhibitor against FVIII and/or FVIIIa.

As used herein, embodiments represented by the expression “comprising ...” include
 5 embodiments represented by the expression “essentially consisting of ...” and embodiments represented by the expression “consisting of ...”.

All patents and reference documents explicitly cited herein are incorporated by reference into this description in their entirety.

The present invention will be further illustrated by the Examples, but it is not to be
 10 construed as being limited thereto.

[Examples]

Herein below, the present invention will be specifically described by the Examples, but it is not to be construed as being limited thereto.

15

[Example 1] Production of antibodies against the anti-FIXa/FX bispecific antibody and sequence determination of the variable region

An attempt was made to generate antibodies against ACE910 (Q499-z121/J327-z119/L404-k) (bispecific antibody in which an H chain consisting of the amino
 20 acid sequence of SEQ ID NO: 9 is associated with the L chain of SEQ ID NO: 10, and an H chain consisting of the amino acid sequence of SEQ ID NO: 11 is associated with the L chain of SEQ ID NO: 10), which is a bispecific antibody described in the Patent Document 3 (WO 2012/067176). Gene recombination techniques and pepsin digestion were used to produce F(ab')₂ composed from the respective Fabs of the anti-FIXa side and the anti-FX side.

25 Mice and rats were immunized with anti-FIXa-F(ab')₂ or anti-FX-F(ab')₂. Cells obtained from the spleen removed from the mice or rats or from rat lymph nodes were subjected to cell fusion with mouse myeloma cells by following general methods to produce the hybridomas. The culture supernatants of the hybridomas were evaluated by ELISA which detects the binding of ACE910 to the anti-FIXa-arm or the anti-FX-arm, and ultimately, mouse
 30 antibodies, AQ8 and AQ1, and rat antibody, AQ512, which bind only to the anti-FIXa-arm but not to the anti-FX-arm of ACE910, and rat antibodies, AJ540, AJ114, AJ521, AJ522, and AJ541, which bind only to the anti-FX-arm but not to the anti-FIXa-arm of ACE910 were selected. In addition, the nucleotide sequences of the variable regions of the AQ8 antibody or the AJ540 antibody were analyzed. The nucleotide sequences of the variable regions of AQ8 and AJ540,
 35 and amino acid sequences predicted therefrom were analyzed using GENETYX Ver.9 (GENETYX CORPORATION).

[Example 2] Production of expression vectors for recombinant mouse antibody AQ8 and recombinant rat-rabbit chimeric antibody AJ540.

Recombinant mouse antibody AQ8 was prepared by combining the variable region sequences of the AQ8 antibody obtained in Example 1 with a known mouse IgG2b constant region sequences (heavy chain: EMBL accession No. J00461; light chain: EMBL accession No. V00807) to produce the full-length antibody gene, and then inserting it into an expression vector. Similarly, a recombinant rat-rabbit chimeric antibody AJ540 was produced by combining a known rabbit IgG (heavy chain: EMBL accession No. L29172, light chain: EMBL accession No. X00231) with the variable regions of the AJ540 antibody. The produced expression clone plasmids were introduced into HEK293 cells, large-scale culturing and purification with Protein A and gel filtration were performed, and recombinant mouse antibody AQ8 (rAQ8-mIgG2b) and recombinant rat-rabbit chimeric antibody AJ540 (rAJ540-rbtIgG) were produced.

[Example 3] One-stage clotting assay carried out under neutralization of the anti-FIXa/FX bispecific antibody using rAQ8-mIgG2b and rAJ540-rbtIgG

To FVIII-deficient plasma (George King) containing 10 U/dL or 100 U/dL recombinant FVIII (Kogenate FS, Bayer Yakuhin, Ltd.), the anti-FIXa/FX bispecific antibody ACE910 was added at 0 µg/mL or 300 µg/mL. Furthermore, each of the prepared plasma samples was divided into the following two groups to prepare measurement sample solutions: a group subjected to ten-fold dilution using an imidazole buffer (Kyowa Medex); and a group subjected to ten-fold dilution using an imidazole buffer supplemented with 300 µg/mL each of rAQ8-mIgG2b and rAJ540-rbtIgG. Amounts of rAQ8-mIgG2b and rAJ540-rbtIgG required to sufficiently neutralize ACE910 were added. Details of the combinations are shown below.

[Table 1]

Sample No.	Plasma		Dilution buffer
	Type	Dilution rate	
#1	FVIII deficient plasma containing 10 U/dL recombinant FVIII	10-fold	Imidazole buffer
#2		10-fold	Imidazole buffer supplemented with rAQ8-mIgG2b and rAJ540-rbtIgG
#3	FVIII deficient plasma containing 10 U/dL recombinant FVIII	10-fold	Imidazole buffer
#4		10-fold	Imidazole buffer supplemented

	supplemented with 300 µg/mL anti-FIXa/FX bispecific antibody		with rAQ8-mIgG2b and rAJ540-rbtIgG
#5	FVIII deficient plasma containing	10-fold	Imidazole buffer
#6	100 U/dL recombinant FVIII	10-fold	Imidazole buffer supplemented with rAQ8-mIgG2b and rAJ540-rbtIgG
#7	FVIII deficient plasma containing	10-fold	Imidazole buffer
#8	100 U/dL recombinant FVIII supplemented with 300 µg/mL anti-FIXa/FX bispecific antibody	10-fold	Imidazole buffer supplemented with rAQ8-mIgG2b and rAJ540-rbtIgG

Furthermore, to produce a calibration curve for conversion of coagulation time to FVIII activity, solutions of standard plasma, Coagtrol N (Sysmex), were prepared by performing 10-fold, 20-fold, 40-fold, 80-fold, and 160-fold dilutions using an imidazole buffer (FVIII activities for the respective calibration curve solutions were specified as 93%, 46.5%, 23.3%, 11.6%, and 5.81%). Fifty microliters of a measurement sample solution or calibration curve solution, 50 µL of factor VIII-deficient human plasma (Sysmex), and 50 µL of Thrombocheck APTT-SLA (Sysmex) were mixed and incubated at 37°C for five minutes. After incubation, 50 µL of 0.02 mol/L calcium chloride solution (Sysmex) was added to initiate coagulation, and the coagulation time was measured using automatic blood coagulation analyzer KC4 Delta (Stago).

Coagulation time of a measurement sample was converted to FVIII activity according to the coagulation time at each FVIII activity of the calibration curve solution.

Results

The results are shown in Fig. 1. When FVIII-deficient plasma containing 10 U/dL or 100 U/dL recombinant FVIII supplemented with an anti-FIXa/FX bispecific antibody was diluted with a buffer (#3, #7), the FVIII activities were shown to be above the range of the calibration curve, and could not be accurately measured. On the other hand, when FVIII-deficient plasma containing 10 U/dL or 100 U/dL recombinant FVIII supplemented with an anti-FIXa/FX bispecific antibody was diluted with a buffer containing two types of antibodies against the anti-FIXa/FX bispecific antibody (#4, #8), the FVIII activities were shown to be similar to those of the groups without addition of the anti-FIXa/FX bispecific antibody (#1, #5). Therefore, this shows that the antibodies against the anti-FIXa/FX bispecific antibody completely neutralized the activity of the bispecific antibody to enable accurate measurement of the FVIII activity in plasma even in the presence of the bispecific antibody. When

FVIII-deficient plasma containing 10 U/dL or 100 U/dL recombinant FVIII was diluted with a buffer containing only the two types of antibodies against the anti-FIXa/FX bispecific antibody (#2, #6), the FVIII activities were shown to be similar to those of the groups without addition of the anti-FIXa/FX bispecific antibody (#1, #5); therefore, antibodies against the anti-FIXa/FX bispecific antibody were found to have neutralizing effects specific to the bispecific antibody.

[Example 4] One-stage clotting assay carried out under neutralization of the anti-FIXa/FX bispecific antibody using AQ1 and AJ541 or AQ1 and AJ522

To FVIII-deficient plasma (George King) containing 10 U/dL recombinant FVIII (Kogenate FS, Bayer Yakuhin, Ltd.), the anti-FIXa/FX bispecific antibody ACE910 was added at 0 µg/mL or 10 µg/mL. Furthermore, each of the prepared plasma was divided into three groups to prepare measurement sample solutions: a group subjected to ten-fold dilution using an imidazole buffer (Kyowa Medex); a group subjected to ten-fold dilution using an imidazole buffer supplemented with 100 µg/mL each of AQ1 and AJ541; and a group subjected to ten-fold dilution using an imidazole buffer supplemented with 100 µg/mL each of AQ1 and AJ522. Amounts of AQ1, AJ541 and AJ522 required to sufficiently neutralize ACE910 were added. Details of the combinations are shown below.

[Table 2]

Sample No.	Plasma		Dilution buffer
	Type	Dilution rate	
#1	FVIII deficient plasma containing 10 U/dL recombinant FVIII	10-fold	Imidazole buffer
#2		10-fold	Imidazole buffer supplemented with AQ1 and AJ541
#3		10-fold	Imidazole buffer supplemented with AQ1 and AJ522
#4	FVIII-deficient plasma containing 10 U/dL recombinant FVIII supplemented with 10 µg/mL ACE910	10-fold	Imidazole buffer
#5		10-fold	Imidazole buffer supplemented with AQ1 and AJ541
#6		10-fold	Imidazole buffer supplemented with AQ1 and AJ522

Furthermore, to produce a calibration curve for conversion of coagulation time to FVIII activity, solutions of standard plasma, Coagtrol N (Sysmex), were prepared by performing

10-fold, 20-fold, 40-fold, 80-fold, 160-fold, 320-fold and 640-fold dilutions using an imidazole buffer (FVIII activities for the respective calibration curve solutions were specified as 102%, 51.0%, 25.5%, 12.8%, 6.38%, 3.19% and 1.59%). Fifty microliters of a measurement sample solution or calibration curve solution, 50 μ L of factor VIII-deficient human plasma (Sysmex), and 50 μ L of Thrombocheck APTT-SLA (Sysmex) were mixed and incubated at 37°C for five minutes. After incubation, 50 μ L of 0.02 mol/L calcium chloride solution (Sysmex) was added to initiate coagulation, and the coagulation time was measured using automatic blood coagulation analyzer KC4 Delta (Stago).

Coagulation time of a measurement sample was converted to FVIII activity according to the coagulation time at each FVIII activity of the calibration curve solution.

Results

The results are shown in Fig. 2. When FVIII-deficient plasma containing 10 U/dL recombinant FVIII supplemented with an anti-FIXa/FX bispecific antibody, ACE910, was diluted with a buffer (#4), the FVIII activity was shown to be above the range of the calibration curve, and could not be accurately measured. On the other hand, when FVIII-deficient plasma containing 10 U/dL recombinant FVIII supplemented with an anti-FIXa/FX bispecific antibody, ACE910, was diluted with a buffer containing two types of antibodies, AQ1 and AJ541, against the anti-FIXa/FX bispecific antibody (#5) or a buffer containing two types of antibodies, AQ1 and AJ522, against the anti-FIXa/FX bispecific antibody (#6), the FVIII activity was shown to be similar to that of the group without addition of the anti-FIXa/FX bispecific antibody (#1). Therefore, this shows that not only rAQ8-mIgG2b and rAJ540-rbIgG, but other antibody combinations are also effective as antibodies against the anti-FIXa/FX bispecific antibody to completely neutralize the activity of the bispecific antibody ACE910.

[Example 5] One-stage clotting assay carried out under neutralization of the anti-FIXa/FX bispecific antibody using AQ512 and AJ114 or AQ512 and AJ521

To FVIII-deficient plasma (George King) containing 10 U/dL recombinant FVIII (Kogenate FS, Bayer Yakuhin, Ltd.), the anti-FIXa/FX bispecific antibody hBS23 was added at 0 μ g/mL or 10 μ g/mL. Furthermore, each of the prepared plasma was divided into three groups to prepare measurement sample solutions: a group subjected to ten-fold dilution using an imidazole buffer (Kyowa Medex); a group subjected to ten-fold dilution using an imidazole buffer supplemented with 100 μ g/mL each of AQ512 and AJ114; and a group subjected to ten-fold dilution using an imidazole buffer supplemented with 100 μ g/mL each of AQ512 and AJ521. Amounts of AQ512, AJ114, and AJ521 required to sufficiently neutralize hBS23 were added. Details of the combinations are shown below.

[Table 3]

Sample No.	Plasma		Dilution buffer
	Type	Dilution rate	
#1	FVIII-deficient plasma containing 10 U/dL recombinant FVIII	10-fold	Imidazole buffer
#2		10-fold	Imidazole buffer supplemented with AQ512 and AJ114
#3		10-fold	Imidazole buffer supplemented with AQ512 and AJ521
#4	FVIII-deficient plasma containing 10 U/dL recombinant FVIII supplemented with 10 µg/mL hBS23	10-fold	Imidazole buffer
#5		10-fold	Imidazole buffer supplemented with AQ512 and AJ114
#6		10-fold	Imidazole buffer supplemented with AQ512 and AJ521

Furthermore, to produce a calibration curve for conversion of coagulation time to FVIII activity, solutions of standard plasma, Coagtrol N (Sysmex), were prepared by performing 10-fold, 20-fold, 40-fold, 80-fold, 160-fold, 320-fold and 640-fold dilutions using an imidazole buffer (FVIII activities for the respective calibration curve solutions were specified as 102%, 51.0%, 25.5%, 12.8%, 6.38%, 3.19% and 1.59%). Fifty microliters of a measurement sample solution or calibration curve solution, 50 µL of factor VIII-deficient human plasma (Sysmex), and 50 µL of Thrombocheck APTT-SLA (Sysmex) were mixed and incubated at 37°C for five minutes. After incubation, 50 µL of 0.02 mol/L calcium chloride solution (Sysmex) was added to initiate coagulation, and the coagulation time was measured using automatic blood coagulation analyzer KC4 Delta (Stago).

Coagulation time of a measurement sample was converted to FVIII activity according to the coagulation time at each FVIII activity of the calibration curve solution.

Results

The results are shown in Fig. 3. When FVIII-deficient plasma containing 10 U/dL recombinant FVIII supplemented with an anti-FIXa/FX bispecific antibody, hBS23, was diluted with a buffer (#4), the FVIII activity was shown to be above the range of the calibration curve, and could not be accurately measured. On the other hand, when FVIII-deficient plasma containing 10 U/dL recombinant FVIII supplemented with an anti-FIXa/FX bispecific antibody,

hBS23, was diluted with a buffer containing two types of antibodies, AQ512 and AJ114, against the anti-FIXa/FX bispecific antibody (#5) or a buffer containing two types of antibodies, AQ512 and AJ521, against the anti-FIXa/FX bispecific antibody (#6), the FVIII activity was shown to be similar to that of the group without addition of the anti-FIXa/FX bispecific antibody (#1).

- 5 These show that even with hBS23, a bispecific antibody different from ACE910, the FVIII activity in plasma can be accurately measured despite the presence of the bispecific antibody by completely neutralizing its activity, and therefore the present approach is effective for various bispecific antibodies that have FVIII-substituting activity.

- 10 [Example 6] Bethesda assay carried out under neutralization of the anti-FIXa/FX bispecific antibody using rAQ8-mIgG2b and rAJ540-rbtIgG

To factor VIII-deficient human plasma (containing FVIII inhibitors) (George King Bio-Medical), anti-FIXa/FX bispecific antibody ACE910 was added at 0 $\mu\text{g/mL}$ or 300 $\mu\text{g/mL}$. Furthermore, each of the prepared plasma samples was subjected to 25-fold dilution or 30-fold
 15 dilution using a 0.25% (w/v) bovine serum albumin (Sigma-Aldrich)-containing imidazole buffer (Kyowa Medex) (hereinafter referred to as BSA-imidazole). To Coagtrol N (Sysmex) which is standard plasma, rAQ8-mIgG2b and rAJ540-rbtIgG were either not added, or they were added at 300 $\mu\text{g/mL}$ each.

- Two types of the prepared plasma samples were mixed in equal amounts in the
 20 following combinations (a total of 8 types), and then subjected to incubation at 37°C for two hours.

[Table 4]

Sample No.	Plasma 1		Plasma 2
	Type	Dilution rate	
#1	Factor VIII-deficient human plasma (containing inhibitors) without addition of the anti-FIXa/FX bispecific antibody	25-fold	Coagtrol N without addition of rAQ8-mIgG2b and rAJ540-rbtIgG
		30-fold	
#2		25-fold	Coagtrol N containing 300 $\mu\text{g/mL}$ rAQ8-mIgG2b and 300 $\mu\text{g/mL}$ rAJ540-rbtIgG
		30-fold	
#3	Factor VIII-deficient human plasma (containing inhibitors)	25-fold	Coagtrol N without addition of rAQ8-mIgG2b and rAJ540-rbtIgG
		30-fold	
#4	containing 300 $\mu\text{g/mL}$ anti-FIXa/FX bispecific antibody	25-fold	Coagtrol N containing 300 $\mu\text{g/mL}$ rAQ8-mIgG2b and 300 $\mu\text{g/mL}$ rAJ540-rbtIgG
		30-fold	

After incubation, the mixed solutions were further diluted ten-fold with BSA-imidazole to prepare measurement sample solutions. Furthermore, to prepare a calibration curve for conversion of coagulation time to FVIII activity values, solutions were prepared by diluting Coagtrol N with BSA-imidazole at 20-fold, 40-fold, 80-fold, 160-fold, and 320-fold dilution (FVIII activities of the respective calibration curve solutions were specified as 100%, 50%, 25%, 12.5%, and 6.25%).

Fifty microliters of a measurement sample solution or calibration curve solution, 50 μ L of factor VIII-deficient human plasma (Sysmex), and 50 μ L of Thrombocheck APTT-SLA (Sysmex) were mixed and incubated at 37°C for three minutes. After incubation, 50 μ L of 0.02 mol/L calcium chloride solution (Sysmex) was added to initiate coagulation, and the coagulation time was measured using automatic blood coagulation analyzer KC4 Delta (Stago).

Coagulation time of a measurement sample was converted to FVIII activity according to the coagulation time at each FVIII activity of the calibration curve solution. Furthermore, when the residual FVIII activity was 50%, this was specified as 1 Bethesda, and after calculating the Bethesda values in the measurement sample, mean value calculated by multiplying the value by 25 or 30 was determined as the inhibitor titer in each of the original sample solutions.

Results

The results are shown in Fig. 4. The FVIII inhibitor plasma containing only the anti-FIXa/FX bispecific antibody (#3) showed an activity that was 100% or more of FVIII of the calibration curve; therefore, the FVIII inhibitor titer could not be determined.

On the other hand, FVIII inhibitor plasma containing the anti-FIXa/FX bispecific antibody and the two types of antibodies against the anti-FIXa/FX bispecific antibody (#4) showed an FVIII inhibitor titer similar to that of the inhibitor plasma without additives (#1). Therefore, this shows that the antibodies against the anti-FIXa/FX bispecific antibody completely neutralized the activity of the bispecific antibody to enable accurate measurement of the FVIII inhibitor titer in plasma even in the presence of the bispecific antibody. FVIII inhibitor plasma containing only the two types of antibodies against the anti-FIXa/FX bispecific antibody (#2) showed similar results to that of #1; therefore, antibodies against the anti-FIXa/FX bispecific antibody were found to have neutralizing effects specific to the bispecific antibody.

Industrial Applicability

The present invention provides methods for measuring the reactivity of FVIII in the presence of a bispecific antibody having an activity of functionally substituting for FVIII, for example, methods for measuring FVIII activity or FVIII inhibitor titer. Use of the methods of

the present invention enables accurate measurement of the reactivity of FVIII in patients during treatment of hemorrhagic diseases, such as hemophilia, by using the bispecific antibody.

CLAIMS

1. A method for measuring reactivity of coagulation factor VIII, wherein the method comprises
5 the step of contacting
 (1) a blood-derived sample containing a substance that has an activity of functionally
substituting for coagulation factor VIII, with
 (2) one or more substances that neutralize the substance having an activity of functionally
substituting for coagulation factor VIII.
10
2. The method of claim 1, wherein the substance having an activity of functionally substituting
for coagulation factor VIII is a bispecific antibody that binds to coagulation factor IX and/or
activated coagulation factor IX and to coagulation factor X and/or activated blood coagulation
factor X.
15
3. The method of claim 1 or 2, wherein the bispecific antibody is any one of the antibodies
described below, in which a first polypeptide is associated with a third polypeptide and a second
polypeptide is associated with a fourth polypeptide:
 a bispecific antibody in which the first polypeptide is an H chain consisting of the amino acid
20 sequence of SEQ ID NO: 9, the second polypeptide is an H chain consisting of the amino acid
sequence of SEQ ID NO: 11, and the third polypeptide and the fourth polypeptide are common L
chains of SEQ ID NO: 10 (Q499-z121/J327-z119/L404-k); or
 a bispecific antibody in which the first polypeptide is an H chain consisting of the amino acid
sequence of SEQ ID NO: 36, the second polypeptide is an H chain consisting of the amino acid
25 sequence of SEQ ID NO: 37, and the third polypeptide and the fourth polypeptide are common L
chains of SEQ ID NO: 38 (Q153-G4k/J142-G4h/L180-k).
4. The method of any one of claims 1 to 3, wherein the neutralizing substance is one or more
substances selected from the group consisting of peptides, polypeptides, organic compounds,
30 aptamers, and antibodies that neutralize the substance having an activity of functionally
substituting for coagulation factor VIII.
5. The method of any one of claims 2 to 4, wherein the neutralizing substance is one or more
antibodies selected from the group consisting of an antibody that binds to Fab comprising an
35 antigen-binding site that binds to coagulation factor IX, an antibody that binds to Fab comprising
an antigen-binding site that binds to activated coagulation factor IX, an antibody that binds to

- Fab comprising an antigen-binding site that binds to coagulation factor X, an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor X, and a bispecific antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX and/or activated coagulation factor IX and Fab comprising an antigen-binding site that binds to coagulation factor X and/or activated coagulation factor X.
- 5
6. The method of any one of claims 1 to 5, wherein the neutralizing substance is one or more combinations selected from the group consisting of the following antibody combinations:
- (a) an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX and an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X;
- 10 (b) an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX and an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X;
- 15 (c) an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX and an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX; and
- (d) an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX, an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X, and an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX.
- 20
7. The method of any one of claims 1 to 6, wherein the method for measuring reactivity of coagulation factor VIII is a method for measuring the coagulation factor VIII activity or a method for measuring the coagulation factor VIII inhibitor titer.
- 25
8. A kit for use in the method of any one of claims 1 to 7, wherein the kit comprises one or more antibodies selected from the group consisting of an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX, an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX, an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X, an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor X, and a bispecific antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX and/or activated coagulation factor IX and Fab comprising an antigen-binding site that binds to coagulation factor X and/or activated coagulation factor X.
- 30
- 35

9. The kit of claim 8, wherein the kit comprises one or more combinations selected from the group consisting of the following antibody combinations:
- (a) an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX and an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X;
 - (b) an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX and an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X;
 - (c) an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX and an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX; and
 - (d) an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor IX, an antibody that binds to Fab comprising an antigen-binding site that binds to coagulation factor X, and an antibody that binds to Fab comprising an antigen-binding site that binds to activated coagulation factor IX.
10. A method for diagnosing the disease severity of a patient administered with a substance having an activity of functionally substituting for coagulation factor VIII, wherein the method uses the method of any one of claims 1 to 7.
11. A method for diagnosing inhibitor titer in a patient administered with a substance having an activity of functionally substituting for coagulation factor VIII, wherein the method uses the method of any one of claims 1 to 7.
12. A method for monitoring pharmacological activity of an FVIII formulation in a patient administered with the FVIII formulation and a substance having an activity of functionally substituting for coagulation factor VIII, wherein the method uses the method of any one of claims 1 to 7.
13. The method of any one of claims 10 to 12, wherein the patient is a patient selected from the group consisting of a hemophilia A patient, an acquired hemophilia A patient, a von Willebrand disease patient, and a patient with hemophilia A in which an inhibitor against blood coagulation factor VIII and/or activated blood coagulation factor VIII emerges.
14. The kit of claim 8 or 9, wherein the kit is for diagnosing the disease severity of a patient administered with a substance having an activity of functionally substituting for coagulation

factor VIII.

15. The kit of claim 8 or 9, wherein the kit is for diagnosing inhibitor titer in a patient administered with a substance having an activity of functionally substituting for coagulation factor VIII.

16. The kit of claim 8 or 9, wherein the kit is for monitoring pharmacological activity of an FVIII formulation in a patient administered with the FVIII formulation and a substance having an activity of functionally substituting for coagulation factor VIII.

10

17. The kit of any one of claims 14 to 16, wherein the patient is a patient selected from the group consisting of a hemophilia A patient, an acquired hemophilia A patient, a von Willebrand disease patient, and with a patient with hemophilia A in which an inhibitor against blood coagulation factor VIII and/or activated blood coagulation factor VIII emerges.

15

SEQUENCE LISTING

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CHUGAI SEIYAKU KABUSHIKI KAISHA

<120> Method for measuring reactivity of FVIII

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<151> 2014-09-26

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Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
 115 120 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
 130 135 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
 145 150 155 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
 165 170 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
 180 185 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
 195 200 205

Phe Asn Arg Gly Glu Cys
 210

<210> 11
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 <213> Artificial

<220>
 <223> Artificial sequence

<400> 11

Gln Val Gln Leu Val Gln Ser Gly Ser Glu Leu Lys Lys Pro Gly Ala
 1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Asn
 20 25 30

Asn Met Asp Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met

	35		40		45												
Gly	Asp	Ile	Asn	Thr	Arg	Ser	Gly	Gly	Ser	Ile	Tyr	Asn	Glu	Glu	Phe		
	50					55					60						
Gln	Asp	Arg	Val	Ile	Met	Thr	Val	Asp	Lys	Ser	Thr	Asp	Thr	Ala	Tyr		
65					70					75					80		
Met	Glu	Leu	Ser	Ser	Leu	Arg	Ser	Glu	Asp	Thr	Ala	Thr	Tyr	His	Cys		
				85					90					95			
Ala	Arg	Arg	Lys	Ser	Tyr	Gly	Tyr	Tyr	Leu	Asp	Glu	Trp	Gly	Glu	Gly		
			100					105					110				
Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val	Phe		
	115						120					125					
Pro	Leu	Ala	Pro	Cys	Ser	Arg	Ser	Thr	Ser	Glu	Ser	Thr	Ala	Ala	Leu		
	130					135					140						
Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp		
145				150					155						160		
Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu		
				165				170						175			
Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser		
			180				185						190				
Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Thr	Cys	Asn	Val	Asp	His	Lys	Pro		
		195				200						205					

Ser Asn Thr Lys Val Asp Lys Arg Val Glu Ser Lys Tyr Gly Pro Pro
 210 215 220

Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu Gly Gly Pro Ser Val Phe
 225 230 235 240

Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
 245 250 255

Glu Val Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro Glu Val
 260 265 270

Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
 275 280 285

Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
 290 295 300

Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
 305 310 315 320

Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser
 325 330 335

Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
 340 345 350

Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
 355 360 365

Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
 370 375 380

Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
 385 390 395 400

Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
 405 410 415

Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
 420 425 430

Asn His Tyr Thr Gln Glu Ser Leu Ser Leu Ser Pro
 435 440

<210> 12
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 <212> PRT
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<220>
 <223> AQ8 heavy chain CDR1

<400> 12

Asp Tyr Tyr Met Ser
 1 5

<210> 13
 <211> 19
 <212> PRT
 <213> Artificial

<220>
 <223> AQ8 heavy chain CDR2

<400> 13

Leu Ile Arg Asn Lys Ala Asn Gly Tyr Thr Thr Glu Tyr Ser Ala Ser
 1 5 10 15

Val Lys Gly

<210> 14
 <211> 13
 <212> PRT
 <213> Artificial

<220>
 <223> AQ8 heavy chain CDR3

<400> 14

Asp	Ser	Tyr	Tyr	Ser	Tyr	Asp	Gly	Tyr	Ala	Met	Asp	Tyr
1				5					10			

<210> 15
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 <212> DNA
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<220>
 <223> nucleic acid sequence of AQ8 heavy chain CDR1

<400> 15
 gactactaca tgagc

15

<210> 16
 <211> 57
 <212> DNA
 <213> Artificial

<220>
 <223> nucleic acid sequence of AQ8 heavy chain CDR2

<400> 16
 ctgatcagaa acaaggccaa cggctacacc accgagtaca gcgccagcgt gaagggc

57

<210> 17
 <211> 39
 <212> DNA
 <213> Artificial

<220>
 <223> nucleic acid sequence of AQ8 heavy chain CDR3

<400> 17
 gacagctact acagctacga cggctacgcc atggactac

39

<210> 18
 <211> 11
 <212> PRT
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<220>
 <223> AQ8 light chain CDR1

<400> 18
 Gln Ala Ser Glu Asn Ile Tyr Phe Ser Leu Ala
 1 5 10

<210> 19
 <211> 7
 <212> PRT
 <213> Artificial

<220>
 <223> AQ8 light chain CDR2

<400> 19
 Asn Thr Asp Ser Leu Lys Asp
 1 5

<210> 20
 <211> 9

<212> PRT
 <213> Artificial

<220>
 <223> AQ8 light chain CDR3

<400> 20

Arg Gln Ser Tyr Asp Phe Pro Trp Thr
 1 5

<210> 21
 <211> 33
 <212> DNA
 <213> Artificial

<220>
 <223> nucleic acid sequence of AQ8 light chain CDR1

<400> 21
 caggccagcg agaacatcta cttcagcctg gcc 33

<210> 22
 <211> 21
 <212> DNA
 <213> Artificial

<220>
 <223> nucleic acid sequence of AQ8 light chain CDR2

<400> 22
 aacaccgaca gcctgaagga c 21

<210> 23
 <211> 27
 <212> DNA
 <213> Artificial

<220>
 <223> nucleic acid sequence of AQ8 light chain CDR3

<400> 23
cggcagagct acgacttccc ctggacc

27

<210> 24
<211> 5
<212> PRT
<213> Artificial

<220>
<223> AJ540 heavy chain CDR1

<400> 24

Asp Tyr Ala Met His
1 5

<210> 25
<211> 17
<212> PRT
<213> Artificial

<220>
<223> AJ540 heavy chain CDR2

<400> 25

Trp Ile Asn Thr Tyr Thr Gly Lys Pro Thr Tyr Ala Asp Asp Phe Lys
1 5 10 15

Gly

<210> 26
<211> 11
<212> PRT
<213> Artificial

<220>

<223> AJ540 heavy chain CDR3

<400> 26

Glu Gly Gly Gly Tyr Tyr Trp Tyr Phe Asp Phe
1 5 10

<210> 27

<211> 15

<212> DNA

<213> Artificial

<220>

<223> nucleic acid sequence of AJ540 heavy chain CDR1

<400> 27

gactacgccca tgcac

15

<210> 28

<211> 51

<212> DNA

<213> Artificial

<220>

<223> nucleic acid sequence of AJ540 heavy chain CDR2

<400> 28

tggatcaaca cctacaccgg caagcccacc tacgccgacg acttcaaggg c

51

<210> 29

<211> 33

<212> DNA

<213> Artificial

<220>

<223> nucleic acid sequence of AJ540 heavy chain CDR3

<400> 29

gagggcggag gctactactg gtacttcgac ttc

33

<210> 30
 <211> 11
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 <213> Artificial

<220>
 <223> AJ540 light chain CDR1

<400> 30

Lys Ala Asn Gln Asn Val Asp Phe Asn Val Asp
 1 5 10

<210> 31
 <211> 7
 <212> PRT
 <213> Artificial

<220>
 <223> AJ540 light chain CDR2

<400> 31

Lys Ala Ser Asn Arg Tyr Thr
 1 5

<210> 32
 <211> 9
 <212> PRT
 <213> Artificial

<220>
 <223> AJ540 light chain CDR3

<400> 32

Met Gln Ser Asn Ser Phe Pro Leu Thr
 1 5

<210> 33
 <211> 33
 <212> DNA
 <213> Artificial

<220>

<223> nucleic acid sequence of AJ540 light chain CDR1

<400> 33

aaggccaacc agaacgtgga cttcaacgtg gac

33

<210> 34
 <211> 21
 <212> DNA
 <213> Artificial

<220>

<223> nucleic acid sequence of AJ540 light chain CDR2

<400> 34

aaggccagca accggtacac a

21

<210> 35
 <211> 27
 <212> DNA
 <213> Artificial

<220>

<223> nucleic acid sequence of AJ540 light chain CDR3

<400> 35

atgcagagca acagcttccc cctgacc

27

<210> 36
 <211> 448
 <212> PRT
 <213> Artificial

<220>

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<400> 36

Gln Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Tyr Tyr
 20 25 30

Asp Ile Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45

Ala Ser Ile Ser Pro Ser Gly Gly Ser Thr Tyr Tyr Arg Arg Ser Val
 50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr
 65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95

Ala Thr Arg Ala Gly His Asn Tyr Gly Ala Gly Trp Tyr Phe Asp Tyr
 100 105 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
 115 120 125

Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser
 130 135 140

Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
 145 150 155 160

Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe
165 170 175

Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
180 185 190

Thr Val Pro Ser Ser Ser Leu Gly Thr Lys Thr Tyr Thr Cys Asn Val
195 200 205

Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val Glu Ser Lys
210 215 220

Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu Gly Gly
225 230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
245 250 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln Glu
260 265 270

Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
275 280 285

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg
290 295 300

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
305 310 315 320

Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu
325 330 335

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Cys
 340 345 350

Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu
 355 360 365

Trp Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
 370 375 380

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
 385 390 395 400

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp
 405 410 415

Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His
 420 425 430

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu
 435 440 445

<210> 37

<211> 444

<212> PRT

<213> Artificial

<220>

<223> artificial sequence

<400> 37

Gln Val Gln Leu Val Gln Ser Gly Ser Glu Leu Lys Lys Pro Gly Ala
 1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Asn
 20 25 30

Asn Met Asp Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
 35 40 45

Gly Asp Ile Asn Thr Arg Ser Gly Gly Ser Ile Tyr Asn Glu Glu Phe
 50 55 60

Gln Asp Arg Val Thr Met Thr Ile Asp Lys Ser Thr Gly Thr Ala Tyr
 65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95

Ala Arg Arg Arg Ser Tyr Gly Tyr Tyr His Asp Glu Trp Gly Glu Gly
 100 105 110

Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
 115 120 125

Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala Leu
 130 135 140

Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
 145 150 155 160

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu
 165 170 175

Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser
 180 185 190

Ser Ser Leu Gly Thr Lys Thr Tyr Thr Cys Asn Val Asp His Lys Pro
 195 200 205

Ser Asn Thr Lys Val Asp Lys Arg Val Glu Ser Lys Tyr Gly Pro Pro
 210 215 220

Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu Gly Gly Pro Ser Val Phe
 225 230 235 240

Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
 245 250 255

Glu Val Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro Glu Val
 260 265 270

Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
 275 280 285

Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val Val Ser Val
 290 295 300

Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
 305 310 315 320

Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser
 325 330 335

Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
 340 345 350

Ser Gln Cys Glu Met Thr Lys Asn Gln Val Ser Leu Ser Cys Ala Val
 355 360 365

Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
 370 375 380

Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
 385 390 395 400

Gly Ser Phe Phe Leu Val Ser Arg Leu Thr Val Asp Lys Ser Arg Trp
 405 410 415

Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
 420 425 430

Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu
 435 440

<210> 38

<211> 214

<212> PRT

<213> Artificial

<220>

<223> artificial sequence

<400> 38

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Arg Asn Ile Glu Arg Asn
 20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Glu Leu Leu Ile
 35 40 45

Tyr Ser Ala Ser Arg Lys Glu Ser Gly Val Pro Asp Arg Phe Ser Gly
 50 55 60

Ser Arg Tyr Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
 65 70 75 80

Glu Asp Leu Ala Thr Tyr Tyr Cys Gln Gln Tyr Tyr Ser Pro Pro Leu
 85 90 95

Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
 100 105 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
 115 120 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
 130 135 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
 145 150 155 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
 165 170 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
 180 185 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
 195 200 205

Phe Asn Arg Gly Glu Cys
 210