DESCRIPTION

METHOD FOR PRODUCING POLYPEPTIDE HETEROMULTIMER

Technical Field

[0001]

The present invention relates to, for example, a method for producing a polypeptide heteromultimer and a polypeptide heteromultimer having an altered amino acid in an Fc region so as to promote polypeptide heteromultimerization.

Background Art

[0002]

Antibodies have received attention as drugs because of having high stability in blood and few adverse reactions (Non Patent Literatures 1 and 2). Among these antibodies, there exist bispecific antibodies that can each recognize two types of antigens or epitopes at the same time. These bispecific antibodies are expected to have high target specificity and the function of inhibiting a plurality of pathways at the same time (Non Patent Literature 3). For example, already launched catumaxomab is a bispecific antibody binding to an endothelial cell adhesion factor EpCAM and CD3 expressed on T cells, and is used as a therapeutic drug for malignant ascites.

[0003]

Some reports on the production of IgG-type bispecific antibodies give findings about the low efficiency of obtainment of a bispecific antibody of interest or efficient production, albeit with a high degree of difficulty due to difficult purification (Non Patent Literature 3). In the case of transfecting, for example, 4 types in total of genes, i.e., genes of H chains and L chains constituting IgG having two types of variable regions, to cells and secreting these chains by coexpression, the covalent bond between the two types of H chains or the noncovalent bond between the H chain and the L chain occurs at random. Therefore, the ratio of the bispecific antibody of interest is exceedingly low with remarkably reduced production efficiency. A reported approach to solve this problem involves applying amino acid substitution to the CH3 regions of IgG H chains, whereby IgG having different types of H chains in combination can be preferentially secreted (Patent Literature 1 and Non Patent Literatures 4 and 5). This approach is a method which involves substituting an amino acid side chain present in the CH3 region of one H chain with a larger side chain (knob), and substituting its counterpart amino acid side chain present in the CH3 region of another H chain with a smaller side chain (hole) so that the knob is inserted into the hole to promote the heterodimerization of the H chains and to inhibit the

homodimerization of H chains. Also, a method for introducing different charges to the respective CH3 regions of IgG H chains has been reported (Patent Literature 2). Specifically, this method involves substituting an amino acid present in the CH3 region of one H chain with an amino acid having a positive charge, and substituting its counterpart amino acid present in the CH3 region of another H chain with an amino acid having a negative charge to promote the heterodimerization of the H chains and to inhibit the homodimerization of H chains. Meanwhile, a technique of controlling H and L chain pairing has also been reported (Non Patent Literature 6). This approach exploits antibodies prepared by the exchange of an L chain constant region (CL) and an H chain CH1 region in one Fab to efficiently induce the H and L chain pairing of interest. In addition, there also exists an approach using common L chains in both Fabs. In this case, use of the common L chains allows only one type of L chain gene to be introduced into cells, and yields a bispecific antibody without the need of taking H and L chain pairing into consideration. Currently, bispecific antibodies can be formed with high efficiency by combining the H chain heterodimerization technique and the H-L chain pairing control technique. Nevertheless, it is difficult to completely control H and L chain pairing, and a complicated molecular design is required. Another problem is a high degree of difficulty in maintaining the high affinity of the common L chains for two types of antigens.

[0004]

Meanwhile, instead of the gene recombination methods described above, an approach called Fab arm exchange has been reported as a method for preparing a bispecific antibody using monoclonal antibodies separately prepared in advance. This technique has been developed on the basis of the finding that the *in vivo* exchange of an IgG4 half-molecule with a half-molecule of endogenous IgG4 yields a bispecific antibody (BiAb) (Non Patent Literature 7). According to the reports, two types of naturally occurring human IgG4 antibodies are mixed *in vitro* to produce a bispecific antibody (Patent Literature 3), and this reaction occurs more efficiently under a reducing condition (Non Patent Literature 8). Two sites characteristic of IgG4, i.e., amino acid residues at position 228 in the hinge region and at position 409 in the CH3 region have been identified as amino acid residues important for this reaction. It has been found that even in IgG1, the substitution of these two sites with IgG4-type amino acids causes the reaction with efficiency equivalent to that of IgG4 (Patent Literature 4). The Fab arm exchange produces a bispecific antibody of interest by merely mixing *in vitro* monoclonal antibodies prepared by a general method and is thus highly versatile. The half-molecule exchange reaction, however, occurs at random. Therefore, the bispecific antibody obtained by mixing two types of antibodies is theoretically 50% of the total amount of antibodies present in the system. Hence, a method for improving the rate of bispecific antibody formation has been studied. The reaction efficiency can be reportedly improved by introducing asymmetric amino acid alteration

to two types of antibodies, i.e., K409R alteration to the H chains of one antibody and F405L alteration to the H chains of the other antibody, but still remains at approximately 95% (Patent Literature 5 and Non Patent Literature 9). The efficient and stable production of bispecific antibodies inevitably requires convenient purification and minimized lot-to-lot variation. Thus, there has been a demand for the development of an excellent approach that achieves higher reaction efficiency.

Citation List

Patent Literature

[0005]

Patent Literature 1: WO1996/027011
Patent Literature 2: WO2006/106905
Patent Literature 3: WO2005/062916
Patent Literature 4: WO2008/119353
Patent Literature 5: WO2011/131746

Non Patent Literature

[0006]

Non Patent Literature 1: Nat Biotechnol., 23, 1073-1078, 2005
Non Patent Literature 2: Eur J Pharm Biopharm, 59 (3), 389-396, 2005
Non Patent Literature 3: mAbs, 4, 653-663, 2012
Non Patent Literature 4: Protein Engineering, 9, 617-621, 1996
Non Patent Literature 5: Nature Biotechnol., 16, 677-681, 1998
Non Patent Literature 6: Proc. Natl. Acad. Sci., 108, 11187-11192, 2011
Non Patent Literature 7: Immunology, 97, 693-698, 1999
Non Patent Literature 8: Science, 317, 1554-1557, 2007
Non Patent Literature 9: Proc. Natl. Acad. Sci., 110, 5145-5150, 2013

Summary of Invention

Technical Problem

[0007]

The present invention has been made in light of these circumstances, and an object of the present invention is to provide an excellent approach for the efficient and stable production of a heteromultimer with high reaction efficiency, whereby the desired heteromultimer is obtained through the promotion of polypeptide heteromultimerization under a reducing condition.

Solution to Problem

[0008]

The present inventors have conducted diligent studies on a method for controlling the dissociation and association of Fc regions by selecting polypeptides having the Fc regions as polypeptides to be included in a heteromultimer. As a result, the present inventors have found that: the promotion of the dissociation of Fc regions and the control of the association thereof under a reducing condition can be achieved by the substitution of a particular amino acid present in a heavy chain CH3 region; and a desired heteromeric molecule is formed efficiently as compared with the conventional techniques.

[0009]

The present invention is based on these findings and specifically provides the following [1] to [25].

[1] A method for producing a heteromultimer, comprising the steps of:

a) providing a homo variant of first polypeptides each having a first antigen-binding activity and comprising an Fc region;

b) providing a homo variant of second polypeptides each having a second antigen-binding activity different from the first antigen-binding activity and comprising an Fc region;

c) incubating the homo variant of the first polypeptides and the homo variant of the second polypeptides together under a reducing condition that allows cysteines in hinge regions to cause disulfide bond isomerization; and

d) obtaining a heteromultimer comprising the first and second polypeptides, wherein

1 to 3 sets of amino acid residues selected from the following amino acid residue sets:

(1) amino acid residues at EU numbering positions 356 and 439,

(2) amino acid residues at EU numbering positions 357 and 370, and

(3) amino acid residues at EU numbering positions 399 and 409

in a CH3 region contained in the Fc region of the first and/or second polypeptide have the same type of charge, and

when the amino acid residues in the same set among the amino acid residue sets (1) to (3) have the same type of charge as each other both in the CH3 region of the first polypeptide and in the CH3 region of the second polypeptide, the amino acid residues in this set in the CH3 region of the second polypeptide have a charge opposite to that of the amino acid residues in this set in the CH3 region of the first polypeptide.

[2] The method according to [1], wherein the step a) in [1] comprises the step of providing a third polypeptide that forms a multimer with the first polypeptide, and the step b) comprises the step of providing a fourth polypeptide that forms a multimer with the second polypeptide.

[3] The method according to [1] or [2], wherein the amino acid residues having the same type of charge are selected from one or more amino acid residues included in any of the following groups (A) and (B):

(A) glutamic acid (E) and aspartic acid (D); and

(B) lysine (K), arginine (R), and histidine (H).

[4] The method according to any one of [1] to [3], wherein the set(s) of the amino acid residues having the same type of charge as each other in each of the first and second polypeptides is any one of the following amino acid residue sets (1) to (4):

(1) amino acid residues at EU numbering positions 356 and 439,

(2) amino acid residues at EU numbering positions 357 and 370,

(3) amino acid residues at EU numbering positions 399 and 409, and

(4) (i) amino acid residues at EU numbering positions 399 and 409 and

(ii) amino acid residues at EU numbering positions 356 and 439.

[5] The method according to any one of [1] to [4], wherein the set(s) of the amino acid residues having the same type of charge as each other in each of the first and second polypeptides is the following amino acid residue sets:

(i) amino acid residues at EU numbering positions 399 and 409 and

(ii) amino acid residues at EU numbering positions 356 and 439.

[6] The method according to any one of [1] to [5], wherein in the first and/or second polypeptide, an amino acid is altered so as to destabilize the stability of the CH3 region of the first and/or second polypeptide.

[7] The method according to any one of [1] to [6], wherein in the first and/or second polypeptide, an amino acid at EU numbering position 397 and/or 392 is altered.

[8] The method according to any one of [1] to [7], wherein the Fc region of the first and/or second polypeptide is of IgG1, IgG2, IgG3, or IgG4 type.

[9] The method according to any one of [1] to [7], wherein the Fc region of the first and/or second polypeptide is a mouse-derived Fc region.

[10] The method for producing a heteromultimer according to [9], wherein

1 to 3 sets of amino acid residues selected from the following amino acid residue sets:

(1) amino acid residues at EU numbering positions 356 and 439,

(2) amino acid residues at EU numbering positions 360 and 371, and

(3) amino acid residues at EU numbering positions 399 and 409

in the CH3 region contained in the Fc region of the first and/or second polypeptide have the same type of charge, and

when the amino acid residues in the same set among the amino acid residue sets (1) to (3) have the same type of charge as each other both in the CH3 region of the first polypeptide and in

the CH3 region of the second polypeptide, the amino acid residues in this set in the CH3 region of the second polypeptide have a charge opposite to that of the amino acid residues in this set in the CH3 region of the first polypeptide.

[11] A method for producing a heteromultimer, comprising the steps of:

a) providing a homo variant of first polypeptides each having a first antigen-binding activity and comprising an Fc region;

b) providing a homo variant of second polypeptides each having a second antigen-binding activity different from the first antigen-binding activity and comprising an Fc region;

c) incubating the homo variant of the first polypeptides and the homo variant of the second polypeptides together under a reducing condition that allows cysteines in hinge regions to cause disulfide bond isomerization; and

d) obtaining a heteromultimer comprising the first and second polypeptides, wherein

an amino acid at EU numbering position 397 and/or 392 in a CH3 region contained in the Fc region of the first and/or second polypeptide is altered.

[12] The method according to any one of [1] to [11], wherein in the first and/or second polypeptide,

the amino acid at EU numbering position 397 is altered to Met (M), Phe (F), or Tyr (Y), and/or

the amino acid at EU numbering position 392 is altered to Asp (D), Glu (E), Thr (T), Val (V), or Ile (I).

[13] The method according to any one of [1] to [12], wherein in the first and/or second polypeptide, the amino acid at EU numbering position 397 is altered to Phe (F) or Tyr (Y).

[14] The method according to any one of [1] to [13], wherein in the first polypeptide, the amino acid at EU numbering position 356 is altered to Lys (K), and the amino acid at EU numbering position 397 is altered to Phe (F) or Tyr (Y); and in the second polypeptide, the amino acid at EU numbering position 397 is altered to Phe (F) or Tyr (Y), and the amino acid at EU numbering position 439 is altered to Glu (E).

[15] The method according to any one of [1] to [14], wherein the steps a) and b) are carried out by mixing a cell line producing the homo variant of the first polypeptides with a cell line producing the homo variant of the second polypeptides, and the step c) is carried out in the culture supernatant.

[16] The method according to any one of [1] to [15], wherein the heteromultimer is a multispecific antibody or a hetero-Fc fusion protein.

[17] The method according to any one of [1] to [16], wherein the heteromultimer is a bispecific antibody.

[18] The method according to any one of [1] to [17], wherein the step c) described in [1] or [11] involves contact with a reducing agent.

[19] The method according to [18], wherein the step c) involves the addition of an active substance selected from the group consisting of glutathione, L-cysteine, dithiothreitol, β-mercapto-ethanol, TCEP, and 2-MEA.

[20] The method according to [19], wherein the step c) involves the addition of an active substance selected from glutathione and 2-MEA.

[21] A heteromultimer produced by a method according to any one of [1] to [20].

[22] The heteromultimer according to [21], wherein the heteromultimer is a bispecific antibody.

[23] A composition comprising a heteromultimer according to [21] or [22] and a pharmaceutically acceptable carrier.

[24] A heteromultimer comprising a first polypeptide having a first antigen-binding activity and comprising a first Fc region, and a second polypeptide having a second antigen-binding activity different from the first antigen-binding activity and comprising a second Fc region,

the heteromultimer being obtained by incubating a homo variant of the first polypeptides and a homo variant of the second polypeptides together under a reducing condition that allows cysteines in hinge regions to cause disulfide bond isomerization, wherein

1 to 3 sets of amino acid residues in selected from the following amino acid residue sets:

(1) amino acid residues at EU numbering positions 356 and 439,

(2) amino acid residues at EU numbering positions 357 and 370, and

(3) amino acid residues at EU numbering positions 399 and 409

in a CH3 region contained in the Fc region of the first and/or second polypeptide have the same type of charge,

when the amino acid residues in the same set among the amino acid residue sets (1) to (3) have the same type of charge as each other both in the CH3 region of the first polypeptide and in the CH3 region of the second polypeptide, the amino acid residues in this set in the CH3 region of the second polypeptide have a charge opposite to that of the amino acid residues in this set in the CH3 region of the first polypeptide, and

in the first and/or second polypeptide, an amino acid is altered so as to destabilize the stability of the CH3 region of the first and/or second polypeptide.

[25] A heteromultimer produced by a method comprising the steps of:

a) providing a homo variant of first polypeptides each having a first antigen-binding activity and comprising an Fc region;

b) providing a homo variant of second polypeptides each having a second antigen-binding activity different from the first antigen-binding activity and comprising an Fc region;

c) incubating the homo variant of the first polypeptides and the homo variant of the second polypeptides together under a reducing condition that allows cysteines in hinge regions to cause disulfide bond isomerization; and

d) obtaining a heteromultimer comprising the first and second polypeptides, wherein

1 to 3 sets of amino acid residues selected from the following amino acid residue sets:

(1) amino acid residues at EU numbering positions 356 and 439,

(2) amino acid residues at EU numbering positions 357 and 370, and

(3) amino acid residues at EU numbering positions 399 and 409

in a CH3 region contained in the Fc region of the first and/or second polypeptide have the same type of charge,

when the amino acid residues in the same set among the amino acid residue sets (1) to (3) have the same type of charge as each other both in the CH3 region of the first polypeptide and in the CH3 region of the second polypeptide, the amino acid residues in this set in the CH3 region of the second polypeptide have a charge opposite to that of the amino acid residues in this set in the CH3 region of the first polypeptide, and

in the first and/or second polypeptide, an amino acid is altered so as to destabilize the stability of the CH3 region of the first and/or second polypeptide.

Advantageous Effects of Invention

[0010]

According to the present invention, the promotion of the dissociation of Fc regions and the control of the association thereof under a reducing condition can be achieved by the substitution of a particular amino acid present in a heavy chain CH3 region. A production method for efficiently forming a desired heteromeric molecule as compared with the conventional techniques can be provided.

By use of the method of the present invention, convenience in the purification of a bispecific antibody can be improved, and lot-to-lot variation can be minimized, as compared with the conventional techniques.

A feature of the method for producing a heteromultimer according to the present invention is to alter an amino acid residue in a heavy chain CH3 region. Dissociation and association between polypeptides are promoted by introducing the amino acid residue alteration of the present invention into this region. As a result, a desired heteromultimer can be efficiently obtained as compared with the conventional techniques.

Brief Description of Drawings

[0011]

[Figure 1] Figure 1 is a diagram showing results of analyzing a Fab arm exchange reaction product by ion-exchange chromatography. In the diagram, "BiAb" denotes purified bispecific antibody; "H54 homo" denotes a monoclonal antibody having variable regions H54/L28; and "MRA homo" denotes a monoclonal antibody having variable regions MRAH/MRAL. The numeric values indicated by percentage in the diagram represent the rate of bispecific antibody formation and were calculated by dividing the area of a peak corresponding to the bispecific antibody by the area of all antibodies present in the system, followed by multiplication by 100.

[Figure 2] Figure 2 is a diagram showing results of analyzing a Fab arm exchange reaction product by ion-exchange chromatography. This diagram shows results of the reaction under 3 types of reducing conditions using MRAH-G1drP1/MRAL-k0 and H54-G1drN1/L28-k0 as homo variants. The numeric values indicated by percentage in the diagram represent the rate of bispecific antibody formation and were calculated by dividing the area of a peak corresponding to the bispecific antibody by the area of all antibodies present in the system, followed by multiplication by 100.

[Figure 3] Figure 3 is a diagram showing the correlation between the rate of bispecific antibody formation in Fab arm exchange using 5 mM GSH as a reducing agent and the stability of CH3 of the homo variant used. In the diagram, the phrase "Value of higher Tm of CH3 in two types of homo variants" means Tm of CH3 in a homo variant having higher Tm of CH3, i.e., having more stable CH3, between two homo variants used in the reaction.

[Figure 4] Figure 4 is a diagram showing the conformation of human IgG1 (PDB code: 3DO3) at and around V397.

[Figure 5] Figure 5 is a diagram showing the correlation between the rate of bispecific antibody formation in Fab arm exchange using 25 mM 2MEA as a reducing agent and the stability of CH3 of the homo variant used. In the diagram, the phrase "Value of higher Tm of CH3 in two types of homo variants" means Tm of CH3 in a homo variant having higher Tm of CH3, i.e., having more stable CH3, between two homo variants used in the reaction.

[Figure 6] Figure 6 is a diagram showing results of analyzing a Fab arm exchange reaction product by ion-exchange chromatography. This diagram shows results of carrying out the reaction for different reaction times using MRAH-G1dP17/MRAL-k0 and H54-G1dN17/L28-k0 as homo variants. The numeric values indicated by percentage in the diagram represent the rate of bispecific antibody formation and were calculated by dividing the area of a peak corresponding to the bispecific antibody by the area of all antibodies present in the system, followed by multiplication by 100.

[Figure 7] Figure 7 is a diagram showing results of analyzing a Fab arm exchange reaction product by ion-exchange chromatography. This diagram shows results of the reaction in a cell culture supernatant using MRAH-G1mrP1/MRAL-k0 and H54-G1mrN1/L28-k0 as homo

variants. The numeric values indicated by percentage in the diagram represent the rate of bispecific antibody formation and were calculated by dividing the area of a peak corresponding to the bispecific antibody by the area of all antibodies present in the system, followed by multiplication by 100.

[Figure 8] Figure 8 is a diagram showing the conformation of mouse IgG1 (PDB code: 1IGY) at and around the interacting interface between the CH3 domains.

[Figure 9] Figure 9 is a diagram showing results of analyzing a mouse IgG-type Fab arm exchange reaction product by CE-IEF. The numeric values indicated by percentage in the diagram represent the rate of bispecific antibody formation and were calculated by dividing the area of a peak corresponding to the bispecific antibody by the area of all antibodies present in the system, followed by multiplication by 100.

[Figure 10] Figure 10 is a diagram showing the comparison of the cytotoxic activity of an anti-human glypican 3/anti-human CD3 bispecific antibody. A bispecific antibody prepared using human IgG-type Fab arm exchange (Figure 10-1) or mouse IgG-type Fab arm exchange (Figure 10-2) was compared with a bispecific antibody prepared by CrossMab technology.

[Figure 11] Figure 11 is a diagram showing change in concentration in blood of an anti-human glypican 3/anti-human CD3 bispecific antibody prepared by human IgG-type Fab arm exchange and a bispecific antibody prepared by Knobs-into-Holes technology in normal mice.

[Figure 12] Figure 12 is a diagram showing change in concentration in blood of an anti-human IL-6 receptor antibody prepared by mouse IgG-type Fab arm exchange and an anti-human IL-6 receptor antibody having the sequence of naturally occurring mouse IgG1.

Description of Embodiments

[0012]

The present invention relates to a method for producing a desired heteromultimer by altering an amino acid residue in a heavy chain CH3 region in order to promote the dissociation under a reducing condition of the respective homo variants of polypeptides each having a first antigen-binding activity and polypeptides each having a second antigen-binding activity different from the first antigen-binding activity and to control the hetero-association thereof under the reducing condition. The present invention further relates to a method for selecting a desired heteromultimer.

[0013]

Definition of terms

In the present invention, the "polypeptide" refers to a polypeptide (Fc region-containing polypeptide) or a protein (Fc region-containing protein) comprising a heavy chain Fc region in the amino acid sequence. The polypeptide is usually an organism-derived polypeptide, though

the polypeptide of the present invention is not particularly limited thereto. The polypeptide may be, for example, a polypeptide consisting of an artificially designed sequence. Alternatively, a natural polypeptide, a synthetic polypeptide, a recombinant polypeptide, or the like may be used. In addition, fragments of these polypeptides are also included in the polypeptide of the present invention.

[0014]

In the present specification, the "antibody" refers to a natural immunoglobulin or an immunoglobulin produced by partial or complete synthesis. The antibody may be isolated from a natural resource (e.g., plasma or serum containing naturally occurring antibodies) or the culture supernatant of antibody-producing hybridoma cells or may be partially or completely synthesized by use of an approach such as gene recombination. Preferred examples of the antibody include isotypes of immunoglobulins and subclasses of these isotypes. Nine types of classes (isotypes), i.e., IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, and IgM, are known as human immunoglobulins. Four types of classes, i.e., IgG1, IgG2a, IgG2b, and IgG3, are known as mouse immunoglobulins. Of these isotypes, human immunoglobulins IgG1, IgG2, IgG3, and IgG4 and mouse immunoglobulins IgG1, IgG2a, IgG2b, and IgG3 can be included in the antibody of the present invention. IgG1 is preferred as a mouse immunoglobulin. A plurality of allotype sequences based on gene polymorphism are described as human IgG1, human IgG2, human IgG3, and human IgG4 constant regions in Sequences of proteins of immunological interest, NIH Publication No. 91-3242. Any of these sequences can be used in the present invention. Particularly, an amino acid sequence from EU numbering positions 356 to 358 in the sequence of human IgG1 may be DEL or may be EEM. A plurality of allotype sequences based on gene polymorphism are described as a human Igκ (kappa) constant region and a human Igλ (lambda) constant region in Sequences of proteins of immunological interest, NIH Publication No. 91-3242. Any of these sequences can be used in the present invention.

[0015]

The term "Fc region" is used for defining the C-terminal region of an immunoglobulin heavy chain and includes a natural Fc region sequence and a variant Fc region. Although the boundary of the Fc region of an immunoglobulin heavy chain may vary, the Fc region refers to a region comprising hinges or a portion thereof and CH2 and CH3 domains in an antibody molecule. The heavy chain Fc region of human IgG is usually defined as extending from the amino acid residue Cys226 to the carboxyl terminus of the Fc region, though the Fc region of the present invention is not limited thereto. The immunoglobulin Fc region contains two constant regions, i.e., CH2 and CH3. The "CH2" domain of the human IgG Fc region usually extends from amino acid 231 to amino acid 340. The "CH3" domain extends from the carboxyl

terminus of the Fc region to before the CH2 region, i.e., extends from amino acid 341 to about amino acid 447 of IgG.

[0016]

The Fc region can be preferably obtained by the partial digestion of an IgG monoclonal antibody or the like with a proteolytic enzyme such as pepsin followed by the re-elution of a fraction adsorbed on a protein A or protein G column. Such a proteolytic enzyme is not particularly limited as long as the enzyme is capable of digesting a whole antibody so as to restrictively form Fab or F(ab')2 under appropriately set reaction conditions (e.g., pH) of the enzyme. Examples thereof can include pepsin and papain.

[0017]

The position of each alteration site is represented using the EU numbering system (Kabat EA et al., 1991. Sequences of Proteins of Immunological Interest. NIH).

[0018]

In the present invention, the "association" of polypeptides can refer to, for example, a state where two or more polypeptide regions interact with each other.

[0019]

In the present invention, the phrase "controlling association" refers to control so as to attain a desired associated state and more specifically refers to control so as to prevent undesired association between polypeptides (preferably, association between polypeptides having identical amino acid sequences).

[0020]

In the present invention, the "interface" usually refers to the location of association at which polypeptides associate (interact) with each other. Amino acid residues that form the interface are usually one or more amino acid residues contained in the polypeptide regions subjected to this association and are more preferably amino acid residues that are placed close during the association to participate in the interaction. The interaction specifically includes, for example, the case where the amino acid residues that are placed close during the association form a hydrogen bond, an electrostatic interaction, or a salt bridge therebetween.

[0021]

In the present invention, the "homo variant" of polypeptides refers to the associated form of polypeptides having identical amino acid sequences.

[0022]

In the present invention, the "heteromer" of polypeptides refers to the associated form of a first polypeptide and a second polypeptide differing in amino acid sequence by at least one amino acid residue from the first polypeptide.

[0023]

In the present invention, the "dissociation" between polypeptides refers to a state where the associated form of two or more polypeptides in the polypeptide homo variant is separated into the single polypeptides.

[0024]

In the present invention, the "heteromultimer" refers to a protein multimer that is constituted by plural types of polypeptides capable of associating with each other. More specifically, the "heteromultimer" has at least a first polypeptide and a second polypeptide. In this context, the second polypeptide is a molecule differing in amino acid sequence by at least one amino acid residue from the first polypeptide. The heteromultimer preferably has antigen-binding activities against at least two different types of ligands, antigens, receptors, or substrates, etc., though the heteromultimer of the present invention is not particularly limited thereto. The heteromultimer may contain an additional type of polypeptide in addition to the "heterodimer" formed by the first and second polypeptides. Specifically, the "heteromultimer" of the present invention is not limited to the heterodimer and also includes, for example, a heterotrimer and a heterotetramer.

[0025]

In the polypeptide multimer of the present invention comprising the first polypeptide, the second polypeptide, and one or two third polypeptides, the first polypeptide and the second polypeptide can respectively form multimers (dimers) with the third polypeptides. Furthermore, the formed dimers can form a multimer (tetramer) with each other. The two third polypeptides may have completely identical amino acid sequences (which may have a binding activity against the same antigen). Alternatively, the two third polypeptides may have identical amino acid sequences, but have two or more activities (which may have, for example, binding activities against two or more different antigens). In the case of one third polypeptide, this third polypeptide can form a dimer with any one of the first polypeptide and the second polypeptide to form a polypeptide multimer.

[0026]

In the polypeptide multimer of the present invention, the first polypeptide and the second polypeptide preferably have binding activities against different antigens. On the other hand, the third polypeptide may be a polypeptide having a binding activity against the same antigen as that of either of the first polypeptide or the second polypeptide, or both. Alternatively, the third polypeptide may be a polypeptide having a binding activity against an antigen different from that of the first polypeptide and the second polypeptide.

[0027]

Alternatively, the polypeptide multimer of the present invention may be a polypeptide multimer comprising the first polypeptide, the second polypeptide, the third polypeptide, and a

fourth polypeptide. In such a polypeptide multimer, the first polypeptide and the second polypeptide can form multimers (dimers) with the third polypeptide and the fourth polypeptide, respectively. For example, a disulfide bond can be formed between the first polypeptide and the third polypeptide and between the second polypeptide and the fourth polypeptide to form dimers.

In the polypeptide multimer of the present invention, the first polypeptide and the second polypeptide preferably have binding activities against different antigens. On the other hand, the third polypeptide may be a polypeptide having a binding activity against the same antigen as that of either of the first polypeptide or the second polypeptide, or both. Alternatively, the third polypeptide may be a polypeptide having a binding activity against an antigen different from that of the first polypeptide and the second polypeptide. The fourth polypeptide may be a polypeptide having a binding activity against the same antigen as that of either of the first polypeptide or the second polypeptide, or both. Alternatively, the fourth polypeptide may be a polypeptide having a binding activity against an antigen different from that of the first polypeptide and the second polypeptide.

[0028]

When the "heteromultimer" according to the present invention is a bispecific antibody, the first polypeptide and the second polypeptide may be, for example, a polypeptide comprising the amino acid sequence of an antibody heavy chain against antigen A and a polypeptide comprising the amino acid sequence of an antibody heavy chain against antigen B, respectively. In this case, the third polypeptide can be a polypeptide comprising the amino acid sequence of an antibody light chain against the antigen A, while the fourth polypeptide can be a polypeptide comprising the amino acid sequence of an antibody light chain against the antigen B.

[0029]

In the present invention, the "polypeptide having an antigen-binding activity" refers to a peptide or a protein of 5 or more amino acids in length having a domain (or region) capable of binding to a protein or a peptide such as an antigen or a ligand, and includes, for example, an antibody heavy chain or light chain variable region, a receptor, a fusion peptide of a receptor and an Fc region, a scaffold, and their fragments. Specifically, the polypeptide having an antigen-binding activity can comprise the amino acid sequence of an antibody variable region, a receptor, a fusion peptide of a receptor and an Fc region, a scaffold, or any of their fragments.

Any polypeptide can be used as the scaffold as long as the polypeptide is conformationally stable and can bind to at least one antigen. Examples of such a polypeptide include, but are not limited to, antibody variable region fragments, fibronectin, protein A domains, LDL receptor A domains, and lipocalin as well as molecules described in Nygren et al. (Current Opinion in Structural Biology, 7: 463-469 (1997); and Journal of Immunol Methods,

290: 3-28 (2004)), Binz et al. (Nature Biotech 23: 1257-1266 (2005)), and Hosse et al. (Protein Science 15: 14-27 (2006)).

Method for obtaining the antibody variable region, the receptor, the fusion peptide of a receptor and an Fc region, the scaffold, and their fragments are well known to those skilled in the art. A polypeptide comprising the amino acid sequence of such a region and the amino acid sequence of an antibody light chain constant region can also be used.

[0030]

In the present invention, the "reducing condition" refers to a condition or an environment where cysteine residues forming an inter-heavy chain disulfide bond in the heavy chain hinge regions are more likely to be reduced than oxidized. The reducing condition preferably refers to a condition or an environment that allows cysteines in hinge regions to cause disulfide bond isomerization between the heavy chains, and particularly preferably refers to a condition or an environment that allows cysteines in heavy chain hinge regions to cause disulfide bond isomerization without causing significant disulfide bond isomerization of cysteines outside the hinge regions (i.e., while conserving the disulfide bond between the heavy chain and the light chain). In the present invention, for example, the time of incubating together the homo variant of the first polypeptides each comprising an Fc region and the second polypeptides each comprising an Fc region under the reducing condition can be appropriately set by those skilled in the art.

[0031]

In the present invention, the "reducing agent" refers to a compound that reduces a molecule in the environment, i.e., a compound that shifts a molecule into a state where the molecule has been more reduced or is being more reduced in the environment. The reducing agent acts by donating an electron so that the reducing agent itself becomes an oxidized state after reduction of a substrate. Thus, the reducing agent is an active substance donating an electron. Examples of the reducing agent include dithiothreitol (DTT), mercaptoethanol, cysteine, thioglycolic acid, cysteamine (2-mercaptoethylamine: 2-MEA), glutathione (GSH), TCEP (tris(2-carboxyethyl)phosphine), and sodium borohydride.

[0032]

In the present invention, the "inter-heavy chain disulfide bond isomerization" refers to the exchange of the disulfide bond, i.e., the reorganization of the disulfide bond, between cysteines contained in different heavy chains.

[0033]

The "disulfide bond formation" refers to the process of forming a covalent bond between two cysteines present in one or two polypeptides. This bond is schematized by "-S--S-".

[0034]

The "reduction of the disulfide bond" refers to the process of cleaving the disulfide bond into two thiol groups (-SH groups).

[0035]

In the present invention, the term "FcγR" or "FcgR" refers to an Fcγ receptor which is a receptor capable of binding to the Fc region of an IgG1, IgG2, IgG3, or IgG4 monoclonal antibody, and means any member of the protein family substantially encoded by Fcγ receptor genes. In humans, this family includes, for example: FcγRI (CD64) including isoforms FcγRIa, FcγRIb, and FcγRIc; FcγRII (CD32) including isoforms FcγRIIa (including allotypes H131 (H type) and R131 (R type)), FcγRIIb (including FcγRIIb-1 and FcγRIIb-2), and FcγRIIc; and FcγRIII (CD16) including isoforms FcγRIIIa (including allotypes V158 and F158) and FcγRIIIb (including allotypes FcγRIIIb-NA1 and FcγRIIIb-NA2); and any yet-to-be-discovered human FcγR or FcγR isoform or allotype. The FcγR includes those derived from humans, mice, rats, rabbits, and monkeys. The FcγR is not limited to these molecules and may be derived from any organism. The mouse FcγRs include, for example, FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16) and FcγRIII-2 (CD16-2), and FcγRIV, and any yet-to-be-discovered mouse FcγR or FcγR isoform or allotype.

[0036]

<u>Method for producing heteromultimer by alteration using charge repulsion of amino acid residues</u>

In a preferred embodiment, the method of the present invention is a method for producing a heteromer of desired polypeptides by altering amino acid residues that form the interface between polypeptides in order to promote the dissociation of the homo variants of the first and second polypeptides for a heteromultimer capable of forming two or more types of multimers and to control association between the polypeptides constituting one or more types of multimers.

[0037]

The polypeptide having a first antigen-binding activity and the polypeptide having a second antigen-binding activity according to the present invention can each comprise the amino acid sequence of an antibody heavy chain constant region or the amino acid sequence of an antibody Fc region. Examples of the amino acid sequence of the antibody Fc region or the antibody heavy chain constant region include, but are not limited to, the amino acid sequences of human IgG-type constant regions or Fc regions. The IgG-type constant regions or Fc regions can be any of naturally occurring isotypes IgG1, IgG2, IgG3, and IgG4. Alternatively, their altered forms may be used. Lysine at EU numbering position 447 and glycine at EU numbering position 446 in the Fc region may be removed by the recombinant gene manipulation of nucleic acids encoding these amino acids.

The polypeptide having a third antigen-binding activity and the polypeptide having a fourth antigen-binding activity according to the present invention can each comprise the amino acid sequence of an antibody light chain constant region. Examples of the amino acid sequence of the antibody light chain constant region can include, but are not limited to, the amino acid sequences of human kappa- and human lambda-type constant regions. Alternatively, their altered forms may be used.

The polypeptide having an antigen-binding activity according to the present invention can comprise the amino acid sequence of an antibody variable region (e.g., the amino acid sequences of CDR1, CDR2, CDR3, FR1, FR2, FR3, and FR4).

[0038]

In a preferred embodiment of the method for controlling dissociation and/or association between polypeptides according to the present invention, examples of the method include a method which involves introducing charge repulsion to the interface between the constant regions of heavy chains to suppress the association between the heavy chains. Examples of the amino acid residues coming in contact with each other at the interface between the heavy chain constant regions can include pairs at positions 356 and 439, at positions 357 and 370, and at positions 399 and 409 in CH3 regions. The sites in the heavy chain constant regions are represented by the EU numbering system.

[0039]

As shown in Examples mentioned later, the method of the present invention is carried out by the alteration of these amino acid residues to control dissociation and/or association between heavy chain polypeptides. As a result, the desired heteromultimer can be preferentially obtained. In a preferred aspect, the present invention provides a polypeptide which is an antibody or an Fc region-containing protein (e.g., an IgG-type antibody, minibody (Alt M et al., FEBS Letters 199, 9; 454: 90-94), and immunoadhesin (Non Patent Literature 2)) comprising two or more types of heavy chain Fc regions, wherein 1 to 3 sets of amino acid residues sets selected from the following amino acid residue sets (1) to (3):

(1) amino acid residues at EU numbering positions 356 and 439,

(2) amino acid residues at EU numbering positions 357 and 370, and

(3) amino acid residues at EU numbering positions 399 and 409

in a first heavy chain Fc region have the same type of charge.

[0040]

The present invention further provides a polypeptide wherein 1 to 3 sets of amino acid residues selected from the amino acid residue sets (1) to (3) in a second heavy chain Fc region different from the first heavy chain Fc region have a charge opposite to that of the counterpart

amino acid residues having the same type of charge as each other in the corresponding set(s) among the amino acid residue sets (1) to (3) in the first heavy chain Fc region.

[0041]

In the polypeptide, the "amino acid residues having a charge" are preferably selected from, for example, amino acid residues included in any of the following groups (a) and (b):

(a) glutamic acid (E) and aspartic acid (D); and

(b) lysine (K), arginine (R), and histidine (H).

[0042]

In the polypeptide, the phrase "having the same type of charge" means that, for example, all of two or more amino acid residues are amino acid residues included in any one of the groups (a) and (b). The phrase "having a charge opposite" means that, for example, when at least one amino acid residue among two or more amino acid residues is an amino acid residue included in any one of the groups (a) and (b), the remaining amino acid residue(s) is an amino acid residue included in the other group.

In a preferred embodiment, the polypeptide may have the cross-link between the first heavy chain CH3 region and the second heavy chain CH3 region through a disulfide bond.

[0043]

In the present invention, examples of the "association interface-controlling alteration" include the following alterations:

(1) the alteration of Asp (D) at EU numbering position 356 in the first heavy chain Fc region to Lys (K), Arg (R), or His (H), and the alteration of Lys (K) at EU numbering position 439 in the second heavy chain Fc region to Glu (E) or Asp (D);

(2) the alteration of Glu (E) at EU numbering position 357 in the first heavy chain Fc region to Lys (K), Arg (R), or His (H), and the alteration of Lys (K) at EU numbering position 370 in the second heavy chain Fc region to Glu (E) or Asp (D); and

(3) the alteration of Asp (D) at EU numbering position 399 in the first heavy chain Fc region to Lys (K), Arg (R), or His (H), and the alteration of Lys (K) at EU numbering position 409 in the second heavy chain Fc region to Glu (E) or Asp (D).

[0044]

In a non-limiting embodiment, the method for controlling dissociation and/or association between polypeptides according to the present invention is associated with a method for producing a mouse heteromultimer. In a preferred embodiment of this method, examples of the method include a method which involves introducing charge repulsion to the interface between the constant regions of heavy chains to suppress the association between the heavy chains. In the method, examples of the amino acid residues coming in contact with each other at the interface between the heavy chain constant regions can include pairs at positions 356 and 439, at

positions 360 and 371, and at positions 399 and 409 in CH3 regions. The sites in the heavy chain constant regions are represented by the EU numbering system.

[0045]

As shown in Examples mentioned later, the method of the present invention is carried out by the alteration of these amino acid residues in the mouse-derived CH3 regions to control dissociation and/or association between heavy chain polypeptides. As a result, the desired heteromultimer can be preferentially obtained. In a preferred aspect, the present invention provides a polypeptide which is an antibody or an Fc region-containing protein (e.g., an IgG-type antibody, minibody (Alt M et al., FEBS Letters 1999; 454: 90-94), and immunoadhesin (Non Patent Literature 2)) comprising two or more types of heavy chain Fc regions, wherein 1 to 3 sets of amino acid residues selected from the following amino acid residue sets (1) to (3):

(1) amino acid residues at EU numbering positions 356 and 439,

(2) amino acid residues at EU numbering positions 360 and 371, and

(3) amino acid residues at EU numbering positions 399 and 409

in a first heavy chain Fc region have the same type of charge.

[0046]

The present invention further provides a polypeptide wherein 1 to 3 sets of amino acid residues selected from the amino acid residue sets (1) to (3) in a second heavy chain Fc region different from the first heavy chain Fc region have a charge opposite to that of the counterpart amino acid residues having the same type of charge as each other in the corresponding set(s) among the amino acid residue sets (1) to (3) in the first heavy chain Fc region.

[0047]

In the polypeptide, the "amino acid residues having a charge" are preferably selected from, for example, amino acid residues included in any of the following groups (a) and (b):

(a) glutamic acid (E) and aspartic acid (D); and

(b) lysine (K), arginine (R), and histidine (H).

[0048]

In the polypeptide, the phrase "having the same type of charge" means that, for example, all of two or more amino acid residues are amino acid residues included in any one of the groups (a) and (b). The phrase "having a charge opposite" means that, for example, when at least one amino acid residue among two or more amino acid residues is an amino acid residue included in any one of the groups (a) and (b), the remaining amino acid residue(s) is an amino acid residue included in the other group.

In a preferred embodiment, the polypeptide may have the cross-link between the first heavy chain CH3 region and the second heavy chain CH3 region through a disulfide bond.

[0049]

In the present invention, examples of the "association interface-controlling alteration" include the following alterations:
(1) the alteration of Asp (D) at EU numbering position 356 in the first heavy chain Fc region to Lys (K), Arg (R), or His (H), and the alteration of Lys (K) at EU numbering position 439 in the second heavy chain Fc region to Glu (E) or Asp (D);
(2) the alteration of Glu (E) at EU numbering position 360 in the first heavy chain Fc region to Lys (K), Arg (R), or His (H), and the alteration of Lys (K) at EU numbering position 371 in the second heavy chain Fc region to Glu (E) or Asp (D); and
(3) the alteration of Asp (D) at EU numbering position 399 in the first heavy chain Fc region to Lys (K), Arg (R), or His (H), and the alteration of Lys (K) at EU numbering position 409 in the second heavy chain Fc region to Glu (E) or Asp (D).

[0050]

The amino acid residues to be "altered" according to the present invention are not limited to the amino acid residues in the polypeptide constant regions. Those skilled in the art can find amino acid residues that form the interface in a polypeptide variant or a heteromultimer by homology modeling or the like using commercially available software, and can alter amino acid residues at the sites so as to control association.

[0051]

The "alteration" of amino acid residues in the method of the present invention specifically refers to, for example, the substitution of the original amino acid residues by other amino acid residues, the deletion of the original amino acid residues, or the addition of a new amino acid residue and preferably refers to the substitution of the original amino acid residues by other amino acid residues.

[0052]

<u>Method for producing heteromultimer by amino acid alteration at position 397 and/or 392</u>

In a more preferred embodiment of the method for controlling dissociation and/or association between polypeptides according to the present invention, the method is a method comprising introducing a mutation of an amino acid residue to a heavy chain Fc region so as to destabilize the stability of the heavy chain CH3 region. This method may further comprise the optional step of introducing the aforementioned amino acid alteration related to interface control using charge repulsion or the like.

[0053]

In the present invention, the "destabilization of the stability of the CH3 region" means that a polypeptide homo variant with at least one or more amino acid residues altered in the Fc region becomes more susceptible to separation into the single polypeptides than the unaltered polypeptide homo variant.

[0054]

In the present invention, the "destabilization of the stability of the CH3 region" preferably means that the intermediate temperature of thermal denaturation (Tm) of the heavy chain CH3 region having the altered amino acid residues at pH 7.4 is 72.5°C or lower, 72.0°C or lower, 71.5°C or lower, 71.0°C or lower, or 70.5°C or lower, more preferably 70.4°C or lower, 70.3°C or lower, 70.2°C or lower, 70.1°C or lower, 70.0°C or lower, 69.9°C or lower, 69.8°C or lower, 69.7°C or lower, 69.6°C or lower, 69.5°C or lower, 69.0°C or lower, 68.5°C or lower, 68.0°C or lower, or 67.5°C or lower.

[0055]

The Tm of the heavy chain CH3 region can be measured by, for example, a method described in Reference Example 3 in the present specification. A buffer solution or the like for use in this measurement can be appropriately selected.

[0056]

In a further preferred embodiment of the method for controlling dissociation and/or association between polypeptides according to the present invention, the method is a method comprising introducing a mutation to an amino acid residue at EU numbering position 397 and/or 392 in a heavy chain CH3 region. This method may further comprise the optional step of introducing the aforementioned amino acid alteration related to interface control using charge repulsion or the like.

[0057]

In a non-limiting embodiment of the present invention, a mutation can also be introduced to an amino acid residue at EU numbering position 397 and/or 392 in a heavy chain CH3 region in the method for controlling dissociation and/or association between mouse-derived polypeptides. This method may further comprise the optional step of introducing the aforementioned amino acid alteration related to interface control using charge repulsion or the like.

[0058]

The amino acid residue for the introduction of a mutation at position 397 is preferably altered to an amino acid having a bulky side chain or an amino acid having a branched side chain.

[0059]

The amino acid residue for the introduction of a mutation at position 392 is preferably altered to an amino acid having a negative charge, an amino acid having a bulky side chain, or an amino acid having a branched side chain.

[0060]

In the present invention, examples of the "amino acid having a bulky side chain" include Met (M), Phe (F), Tyr (Y), Val (V), Leu (L), Ile (I), Trp (W), Arg (R), His (H), Glu (E), Lys (K),

Gln (Q), Asp (D), Asn (N), Cys (C), and Thr (T) and preferably include Met (M), Phe (F), Thr (T), and Tyr (Y).

[0061]

In the present invention, examples of the "amino acid having a branched side chain" include Val (V), Ile (I), and Leu (L) and preferably include Val (V) and Ile (I).

[0062]

In the present invention, examples of the "amino acid having a negative charge" include Asp (D) and Glu (E).

[0063]

In the present invention, preferred examples of the "heteromultimer" can include multispecific antibodies and hetero-fusion proteins.

[0064]

In a non-limiting aspect, the present invention provides the amino acid alteration of a heteromultimer to enhance binding to FcγR. Preferred examples of the amino acid alteration site include, but are not limited to, an amino acid at EU numbering position 397. The amino acid residue for the introduction of a mutation at position 397 is preferably altered to an amino acid having a bulky side chain or an amino acid having a branched side chain.

[0065]

In the present invention, more preferred examples of the multispecific antibody include IgG type, scFv-IgG, Tandem scFv-Fc, DVD-Ig, Diabody-Fc, Single chain Diabody-Fc, IgG-scFv, sVD-IgG, Tandemab, scFv light chain C-terminal fusion, Tri-specific C-terminal fusion, Tri-specific N-terminal fusion, and IgG-Fab (Bispecific Antibodies, Roland E. Kontermann, 2011, WO2010034441, and WO2010145792).

[0066]

In the present invention, the term "antibody" is used in the broadest sense and includes monoclonal antibodies, polyclonal antibodies, and antibody variants (chimeric antibodies, humanized antibodies, low-molecular antibodies (also including antibody fragments), multispecific antibodies, etc.) as long as the antibody exhibits a desired biological activity. In the present invention, the "antibody" may be a polypeptide or may be a heteromultimer. The antibody is preferably a monoclonal antibody, a chimeric antibody, a humanized antibody, or a low-molecular antibody such as an antibody fragment. In the present invention, the method for controlling dissociation and/or association according to the present invention can be preferably used for obtaining (preparing) these antibodies.

[0067]

Preferred examples of the polypeptide or the heteromultimer subjected to the method of the present invention can include a polypeptide or a heteromultimer having an antibody heavy

chain variable region and light chain variable region. In a more preferred aspect, the present invention provides a method for controlling the dissociation and/or association of the polypeptide or the heteromultimer of the present invention comprising two or more types of heavy chain variable regions and two or more types of light chain variable regions.

[0068]

The polypeptide having an antigen-binding activity according to the present invention can comprise the amino acid sequence of an antibody heavy chain or the amino acid sequence of an antibody light chain. More specifically, the polypeptide having a first antigen-binding activity and the polypeptide having a second antigen-binding activity can each comprise the amino acid sequence of an antibody heavy chain. The polypeptide having a third antigen-binding activity and the polypeptide having a fourth antigen-binding activity can each comprise the amino acid sequence of an antibody light chain.

When the polypeptide multimer of interest is a tetramer which is a multimer formed by a dimer formed between the first polypeptide and the third polypeptide and a dimer formed between the second polypeptide and the fourth polypeptide, for example, a polypeptide multimer in which the polypeptides having the first and second antigen-binding activities are polypeptides each comprising the amino acid sequence of an antibody heavy chain while the polypeptides having the third and fourth antigen-binding activities are polypeptides each comprising the amino acid sequence of an antibody light chain can also be used as the polypeptide multimer of the present invention.

[0069]

Further preferred examples of the multispecific antibody of the present invention can include bispecific antibodies.

[0070]

In a preferred aspect of the present invention, the present invention relates to, for example, a method for controlling dissociation and/or association as to a bispecific antibody comprising two types of heavy chains (the first polypeptide and the second polypeptide in the polypeptide multimer according to the present invention) and two types of light chains (the third polypeptide and the fourth polypeptide in the polypeptide multimer according to the present invention).

[0071]

The "bispecific antibody" according to a preferred aspect of the present invention will be described in more detail. The "first polypeptide and the second polypeptide" refer to one (first H chain) of two heavy chains (H chains) constituting the antibody and the other H chain (second H chain) different from the first H chain. In short, any one of the two H chains can be arbitrarily selected as the first H chain, and the other H chain can be set to the second H chain. Likewise, the "third polypeptide and the fourth polypeptide" refer to one (first L chain) of two

light chains (L chains) constituting the bispecific antibody and the other L chain (second L chain) different from the first L chain. Any one of the two L chains can be arbitrarily selected as the first L chain, and the other H chain can be set to the second L chain. Usually, the first L chain and the first H chain are derived from the same antibody that recognizes a certain antigen (or epitope). The second L chain and the second H chain are also derived from the same antibody that recognizes a certain antigen (or epitope). In this context, an L-H chain pair formed by the first H chain and L chain is referred to as a first pair (or first HL molecule). An L-H chain pair formed by the second H chain and L chain is referred to as a second pair (or second HL molecule). The first pair and the second pair may recognize the same antigen and preferably recognize different epitopes. In this case, the H chains or the L chains in the first pair and the second pair preferably have amino acid sequences different from each other. When the first pair and the second pair recognize different epitopes, the first pair may recognize an antigen totally different from that of the second pair, or the first pair and the second pair may recognize different sites (different epitopes) on the same antigen (e.g., when the antigen is a heteromeric receptor, the multispecific antibody recognizes different domains constituting the heteromeric receptor; or when the antigen is a monomer, the multispecific antibody recognizes a plural sites in the monomer antigen). Such a molecule usually binds to two antigens, but may have specificities for two or more (e.g., 3 types of) antigens. Alternatively, one of the pairs may recognize an antigen such as a protein, a peptide, a gene, or a sugar, and the other pair may recognize, for example, a cytotoxic substance such as a radioactive substance, a chemotherapeutic agent, or a cell-derived toxin. In the case of preparing a desired antibody having pairs formed by particular H chains and L chains in combination, the particular H chains and L chains can be arbitrarily determined as the first pair and the second pair.

[0072]

In the present invention, the "fusion protein" refers to a protein in which two or more identical or substantially analogous protein molecules are joined via an Ig hinge region amino acid sequence linker. The prefix "hetero-" is used for describing a fusion protein containing more than one type of proteins. The "hetero-fusion protein" contains, for example, two or more proteins which are one or more residual proteins and one or more different proteins joined together.

[0073]

The "antibody" according to the present invention includes those obtained by further altering the amino acid sequence of the aforementioned antibody by amino acid substitution, deletion, addition and/or insertion, or chimerization, humanization, etc. The alteration of an amino acid sequence by amino acid substitution, deletion, addition and/or insertion, or humanization, chimerization, etc., can be practiced by a method generally known to those skilled

in the art. Likewise, the amino acid sequences of antibody variable regions and constant regions for use in preparing the antibody according to the present invention as a recombinant antibody may be altered by amino acid substitution, deletion, addition and/or insertion, or chimerization, humanization, etc.

[0074]

The antibody according to the present invention may be an antibody derived from any animal, such as a mouse antibody, a human antibody, a rat antibody, a rabbit antibody, a goat antibody, or a camel antibody. The antibody according to the present invention may be an altered antibody prepared by the substitution of the amino acid sequence of, for example, a chimeric antibody, particularly, a humanized antibody. Alternatively, any antibody such as a modified antibody conjugated with various molecules, an antibody fragment, or a low-molecular antibody can be used.

[0075]

The "chimeric antibody" is an antibody prepared from a combination of sequences derived from different animals. Examples thereof can include an antibody composed of heavy chain and light chain variable (V) regions of a mouse antibody and heavy chain and light chain constant (C) regions of a human antibody. The preparation of the chimeric antibody is known in the art. The chimeric antibody can be obtained, for example, by: ligating DNAs encoding the antibody V regions with DNAs encoding the human antibody C regions; incorporating the resulting ligation products into expression vectors; and transferring the vectors into hosts for antibody production.

[0076]

The "humanized antibody", also called reshaped human antibody, is obtained by grafting complementarity-determining regions (CDRs) of an antibody derived from a non-human mammal, for example, a mouse antibody, to CDRs of a human antibody. A method for identifying CDRs is known in the art (Kabat et al., Sequence of Proteins of Immunological Interest (1987), National Institute of Health, Bethesda, Md.; and Chothia et al., Nature (1989) 342: 877). A general gene recombination approach therefor is also known in the art (see European Patent Application Publication No. EP 125023 and WO 96/02576). Accordingly, for example, mouse antibody CDRs are determined by a method known in the art. A DNA encoding an antibody having these CDRs linked to human antibody framework regions (FRs) is obtained. The humanized antibody can be produced in a system using usual expression vectors. Such a DNA can be synthesized by PCR using several oligonucleotide primers prepared so as to have a portion overlapping the terminal regions of both CDR and FR (see a method described in WO98/13388). The human antibody FRs connected via the CDRs are selected such that the CDRs form a favorable antigen-binding site. If necessary, amino acids in the FRs of antibody

variable regions may be altered such that the CDRs of the resulting reshaped human antibody form an appropriate antigen-binding site (Sato et al., Cancer Res. (1993) 53: 851-6). The amino acid residues in the FRs that can be altered include moieties binding directly to an antigen through a noncovalent bond (Amit et al., Science (1986) 233: 747-53), moieties influencing or acting on CDR structures (Chothia et al., J. Mol. Biol. (1987) 196: 901-17), and moieties related to VH-VL interaction (EP239400).

[0077]

When the antibody according to the present invention is a chimeric antibody or a humanized antibody, human antibody-derived constant regions are preferably used as the C regions of the antibody. For example, Cγ1, Cγ2, Cγ3, or Cγ4 can be used for an H chain, and Cκ or Cλ can be used for an L chain. Also, the human antibody C regions may be modified, if necessary, in order to improve the antibody or the stability of its production. The chimeric antibody according to the present invention preferably comprises variable regions of a non-human mammal-derived antibody and constant regions derived from a human antibody. On the other hand, the humanized antibody preferably comprises CDRs of a non-human mammal-derived antibody and FRs and C regions derived from a human antibody. The constant regions derived from a human antibody have amino acid sequences specific for each isotype such as IgG (IgG1, IgG2, IgG3, or IgG4), IgM, IgA, IgD, or IgE. The constant regions used in the humanized antibody according to the present invention may be constant regions of an antibody belonging to any isotype. Preferably, human IgG constant regions are used, though the constant regions according to the present invention are not limited thereto. The FRs derived from a human antibody used in the humanized antibody are not particularly limited and may be derived from an antibody belonging to any isotype.

[0078]

The variable regions and the constant regions of the chimeric antibody or the humanized antibody according to the present invention may be altered by deletion, substitution, insertion and/or addition, etc., as long as the resulting antibody exhibits the binding specificity of the original antibody.

[0079]

The chimeric antibody or the humanized antibody containing a human-derived sequence exhibits reduced antigenicity in a human body and is therefore considered to be useful when administered to humans for a therapeutic purpose or the like.

[0080]

<u>Combination with isoelectric point alternation technique, etc.</u>

In a further preferred embodiment of the present invention, an amino acid mutation that alters the isoelectric point (pI value) of a polypeptide can be introduced to the polypeptide of the

present invention to thereby purify or produce the polypeptide multimer having the first to fourth polypeptides of interest with higher purity and higher efficiency (WO2007114325 and US20130171095). For example, a method for hetero-associating polypeptides comprising two types of heavy chain constant regions by altering the CH3 domains of the heavy chain constant regions (which is described in, e.g., Protein Eng. 1996 Jul; 9 (7): 617-21; Protein Eng Des Sel. 2010 Apr; 23 (4): 195-202; J Biol Chem. 2010 Jun 18; 285 (25): 19637-46; WO2009080254; and US20130195849) and a method for promoting the association of a heavy chain and a light chain in a particular combination (which is described in, e.g., WO2009080251, WO2009080252, and WO2009080253) may be used for the amino acid mutation that is introduced for promoting association between polypeptides.

[0081]

Combination with technique related to target tissue-specific antigen-binding molecule

In a non-limiting embodiment of the present invention, the method of the present invention can be combined with an antibody technique for dissociation from or binding to an antigen in a concentration-dependent manner of a molecule present specifically for a target tissue (WO2013/180200).

[0082]

Combination with other constant region and/or variable region alteration techniques

In a non-limiting embodiment of the present invention, the method of the present invention can be combined with a technique of altering constant regions with the aim of enhancing binding to FcγR (WO2013047752).

[0083]

In an alternative embodiment, examples of the combination of the method of the present invention with other constant region alteration techniques include its combination with a technique of controlling binding to a complement. Any complement component can be used as the complement as long as the complement is a polypeptide that forms a complement cascade. Preferred examples of the complement include complement components C1q, C1r, and C1s involved in the binding of opsonin. An Fc region having a higher binding activity against a complement than that of a naturally occurring Fc region against the complement can be prepared by the amino acid alteration of the naturally occurring Fc region. In this context, the naturally occurring Fc region refers to a human IgG1, IgG2, IgG3, or IgG4 Fc region. Whether or not the Fc region has a higher binding activity against a complement than that of a naturally occurring Fc region against the complement can be appropriately confirmed by use of an immunological method known in the art such as FACS or ELISA. The term "alteration of amino acid(s)" or "amino acid alteration" of the Fc region includes the alteration of the amino acid sequence of a starting Fc region to a different amino acid sequence. Any Fc region can be

used as the starting domain as long as the resulting modified or altered form of the starting Fc region can bind to the complement in a neutral pH region. An Fc region prepared by further altering an already altered Fc region as a starting Fc region can also be preferably used as the Fc region of the present invention. The starting Fc region can mean the polypeptide itself, a composition containing the starting Fc region, or an amino acid sequence encoding the starting Fc region. The starting Fc region may include an IgG antibody Fc region known in the art, which is produced by the recombination summarized in the section about the antibody. The origin of the starting Fc region is not limited, and the starting Fc region can be obtained from an arbitrary organism of a non-human animal or a human. Preferred examples of the arbitrary organism include organisms selected from mice, rats, guinea pigs, hamsters, gerbils, cats, rabbits, dogs, goats, sheep, cattle, horses, camels, and non-human primates. In another embodiment, the starting Fc region may be obtained from a cynomolgus monkey, a marmoset, a rhesus monkey, a chimpanzee, or a human. Preferably, the starting Fc region can be obtained from human IgG1, but is not limited by the particular class of IgG. This means that a human IgG1, IgG2, IgG3, or IgG4 Fc region can be appropriately used as the starting Fc region. This also means that an Fc region of any IgG class or subclass from the arbitrary organism can be preferably used as the starting Fc region in the present specification. Examples of variants or engineered models of naturally occurring IgG are described in publicly known literatures (Curr. Opin. Biotechnol. (2009) 20 (6), 685-91; Curr. Opin. Immunol. (2008) 20 (4), 460-470; Protein Eng. Des. Sel. (2010) 23 (4), 195-202; and WO2009086320, WO2008092117, WO2007041635, and WO2006105338), though the Fc region according to the present invention is no limited thereto.

[0084]

An amino acid at any position can be altered as long as the amino acid alteration can confer the binding activity against the complement or can enhance binding activity for binding to the complement. The antigen-binding molecule comprising a human IgG1 Fc region as a human Fc region preferably contains the alteration to bring about the effect of enhancing its binding activity against the complement over the binding activity of the starting Fc region of human IgG1. Examples of the amino acid for altering the binding activity against the complement include amino acids in Fc region with altered binding activity against C1q reported in, for example, Duncan et al. (Nature (1988) 332, 738-740), Tao et al. (J. Exp. Med. (1993) 178, 661-667), Brekke et al. (Eur. J. Immunol. (1994) 24, 2542-2547), Xu et al. (Immunol. (1993) 150, 152A), WO1994029351, WO2000042072, and WO2011091078.

[0085]

Examples of such an amino acid that permits the alteration to enhance the binding activity against C1q include at least one or more amino acids selected from EU numbering positions 231

to 238 and positions 318 to 337. One non-limiting example of the amino acid includes at least one or more amino acids selected from the group consisting of positions 235, 237, 267, 268, 276, 318, 320, 322, 324, 327, 331, and 333. The alteration of these amino acids enhances the binding of an IgG-type immunoglobulin Fc region to the complement.

[0086]

Particularly preferred examples of the alteration include the alteration of
an amino acid at EU numbering position 267 to Glu,
an amino acid at EU numbering position 268 to any of Phe and Tyr,
an amino acid at EU numbering position 276 to Arg,
an amino acid at EU numbering position 324 to Thr,
an amino acid at EU numbering position 327 to Gly,
an amino acid at EU numbering position 331 to Pro, or
an amino acid at EU numbering position 333 to any of Ala, Asp, Gly, Ser, and Val
in the Fc region.

The number of amino acids to be altered is not particularly limited. An amino acid at only one site may be altered, or amino acids at two or more sites in arbitrary combination selected from those described above may be altered.

[0087]

In an alternative embodiment, examples of the combination of the method of the present invention with other constant region alteration techniques include its combination with antibody alteration techniques such as an Fc alteration technique of enhancing binding to FcRn at acidic pH (WO2002060919, WO2004035752, and WO2000042072), an Fc alteration technique of enhancing binding to FcRn at neutral pH (WO2011122011 and WO2012133782), a technique of enhancing selective binding to inhibitory Fcγ receptors (WO2012115241 and WO2013125667), a technique of enhancing selective binding to active Fcγ receptors (ADCC activity enhancement technique) (WO2013002362), and a technique of reducing binding activity against rheumatoid factors (WO2013046704).

[0088]

In a non-limiting embodiment, examples of the combination of the method of the present invention with a variable region alteration technique include its combination with alteration techniques such as a pH-dependent antibody (WO2009125825) and a calcium-dependent antibody (WO2012073992).

[0089]

Antibody library, immunization, and hybridoma preparation

A known sequence may be used as a gene encoding the H chain or the L chain of the antibody before the introduction of a mutation (in the present specification, also simply referred

to as the "antibody of the present invention") in the method of the present invention. Alternatively, the gene may be obtained by a method generally known to those skilled in the art. For example, the gene may be obtained from an antibody library or may be obtained by the cloning of an antibody-encoding gene from monoclonal antibody-producing hybridomas.

[0090]

Many antibody libraries have already been known in the art as such an antibody library. Also, methods for preparing the antibody library are known in the art. Thus, those skilled in the art can appropriately obtain the antibody library. For an antibody phage library, for example, see literatures such as Clackson et al., Nature 1991, 352: 624-8, Marks et al., J. Mol. Biol. 1991, 222: 581-97, Waterhouses et al., Nucleic Acids Res. 1993, 21: 2265-6, Griffiths et al., EMBO J. 1994, 13: 3245-60, Vaughan et al., Nature Biotechnology 1996, 14: 309-14, and National Publication of International Patent Application No. 2008-504970. In addition, a method known in the art such as a method for preparing a library using eukaryotic cells (WO95/15393) or a ribosome display method may be used. In addition, a technique of obtaining a human antibody by panning using a human antibody library is also known. For example, human antibody variable regions are expressed as a single-chain antibody (scFv) on the surface of phages by a phage display method. A phage expressing scFv binding to the antigen can be selected. The gene of the selected phage can be analyzed to determine DNA sequences encoding the variable regions of the human antibody binding to the antigen. If the DNA sequence of the scFv binding to the antigen can be determined, appropriate expression vectors can be prepared on the basis of this sequence and used to obtain the human antibody. These methods have already been well known. See WO92/01047, WO92/20791, WO93/06213, WO93/11236, WO93/19172, WO95/01438, and WO95/15388.

[0091]

Basically, a technique known in the art is used in a method for obtaining the antibody-encoding gene from hybridomas. A desired antigen or cells expressing the desired antigen are used as a sensitizing antigen. Animals are immunized with this sensitizing antigen according to a usual immunization method. Immunocytes thus obtained are fused with parental cells known in the art by a usual cell fusion method. Monoclonal antibody-producing cells (hybridomas) are screened for by a usual screening method. From mRNAs of the obtained hybridomas, cDNAs of antibody variable regions (V regions) can be synthesized using reverse transcriptase and ligated with DNAs encoding desired antibody constant regions (C regions) to obtain the antibody-encoding gene.

[0092]

More specifically, although the present invention is not limited by examples below, the sensitizing antigen for obtaining the genes encoding the antibody H chain and L chain includes

both of a complete antigen having immunogenicity and an incomplete antigen (including hapten, etc.) that exhibits no immunogenicity. For example, a full-length protein or a partial peptide of the protein of interest can be used. In addition, a substance constituted by a polysaccharide, a nucleic acid, a lipid, or the like is known to serve as an antigen. The antigen for the antibody of the present invention is not particularly limited. The antigen can be prepared by a method generally known to those skilled in the art and can be obtained according to, for example, a method using baculovirus (e.g., WO98/46777). The hybridomas can be prepared according to, for example, the method of Milstein et al. (G. Kohler and C. Milstein, Methods Enzymol. 1981, 73: 3-46). When the antigen has low immunogenicity, this antigen can be bound to an immunogenic macromolecule such as albumin for immunization. If necessary, the antigen may be bound to another molecule to form a soluble antigen. In the case of using a transmembrane molecule such as a receptor as the antigen, a portion of the extracellular region of the receptor may be used as a fragment, or cells expressing the transmembrane molecule on their surface may be used as the immunogen.

[0093]

The antibody-producing cells can be obtained by the immunization of animals with any of the appropriate sensitizing antigens mentioned above. Alternatively, lymphocytes capable of producing antibodies may be immunized *in vitro* and used as the antibody-producing cells. Various mammals can be used as the animals to be immunized. An animal of the order *Rodentia*, *Lagomorpha*, or *Primates* is generally used. Examples thereof can include: *Rodentia* animals such as mice, rats, and hamsters; *Lagomorpha* animals such as rabbits; and *Primates* animals such as monkeys including cynomolgus monkeys, rhesus monkeys, hamadryas baboons, and chimpanzees. In addition, transgenic animals having repertoires of human antibody genes are also known, and such animals can also be used to obtain the human antibody (see WO96/34096; and Mendez et al., Nat. Genet. 1997, 15: 146-56). Instead of using such transgenic animals, for example, human lymphocytes are sensitized *in vitro* with the desired antigen or cells expressing the desired antigen, and the sensitized lymphocytes can be fused with human myeloma cells, for example, U266, to obtain the desired human antibody having binding activity against the antigen (see Japanese Patent Publication No. 1-59878). Furthermore, transgenic animals having all repertoires of human antibody genes can be immunized with the desired antigen to obtain the desired human antibody (see WO93/12227, WO92/03918, WO94/02602, WO96/34096, and WO96/33735).

[0094]

For the immunization of these animals, for example, the sensitizing antigen is appropriately diluted with or suspended in phosphate-buffered saline (PBS), saline, or the like, mixed with an adjuvant, if necessary, and emulsified. Then, the resulting sensitizing antigen is

intraperitoneally or subcutaneously injected to the animals. Then, the sensitizing antigen, preferably, mixed with a Freund's incomplete adjuvant, is administered to the animals several times at 4- to 21-day intervals. The antibody production can be confirmed by measuring the antibody titer of interest in the serum of the animals by a method routinely used.

[0095]

The hybridomas can be prepared by fusing the antibody-producing cells obtained from the animals or the lymphocytes immunized with the desired antigen with myeloma cells using a fusion agent (e.g., polyethylene glycol) routinely used (Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, 1986, 59-103). If necessary, the hybridoma cells are cultured for growth, and the binding specificity of antibodies produced by the hybridomas is measured by an analysis method known in the art such as immunoprecipitation, radioimmunoassay (RIA), or enzyme-linked immunosorbent assay (ELISA). Then, the hybridoma producing the antibody confirmed by the measurement to have the specificity, affinity, or activity of interest can also be subcloned, if necessary, by an approach such as a limiting dilution method.

[0096]

Subsequently, a gene encoding the selected antibody can be cloned from the hybridoma or the antibody-producing cells (sensitized lymphocytes, etc.) using a probe (e.g., an oligonucleotide complementary to a sequence encoding an antibody constant region) capable of specifically binding to the antibody gene. The gene can also be cloned from mRNA by RT-PCR. Immunoglobulins are classified into five different classes: IgA, IgD, IgE, IgG, and IgM. These classes are further divided into some subclasses (isotypes) (e.g., IgG-1, IgG-2, IgG-3, and IgG-4; and IgA-1 and IgA-2). In the present invention, the H chain and the L chain used in the antibody production can be derived from an antibody belonging to any of these classes and subclasses. Such an antibody is not particularly limited and is particularly preferably IgG.

[0097]

In this context, the genes encoding the H chain and the L chain may be altered by a genetic engineering approach. For example, a generically recombinant antibody, for example, a chimeric antibody or a humanized antibody, can be appropriately prepared by artificially altering an antibody such as a mouse antibody, a rat antibody, a rabbit antibody, a hamster antibody, a sheep antibody, or a camel antibody for the purpose of, for example, reducing hetero-antigenicity in humans. The chimeric antibody is an antibody composed of H chain and L chain variable regions of a non-human mammal antibody, for example, a mouse antibody, and H chain and L chain constant regions of a human antibody. The chimeric antibody can be obtained by: ligating DNAs encoding the mouse antibody variable regions with DNAs encoding the human antibody constant regions; incorporating the resulting ligation products into expression vectors;

and transferring the vectors into hosts for antibody production. The humanized antibody is also called reshaped human antibody. DNA sequences designed to connect complementarity-determining regions (CDRs) of a non-human mammal antibody, for example, a mouse antibody, are synthesized by PCR from several prepared oligonucleotides having overlapping terminal portions. The obtained DNAs are ligated with DNAs encoding human antibody constant regions, and the resulting ligation products are subsequently incorporated to expression vectors, which are then transferred to hosts for antibody production (see EP239400; and WO96/02576). The human antibody FRs connected via the CDRs are selected such that the complementarity-determining regions form a favorable antigen-binding site. If necessary, amino acids in the framework regions of antibody variable regions may be substituted such that the complementarity-determining regions of the resulting reshaped human antibody form an appropriate antigen-binding site (K. Sato et al., Cancer Res. 1993, 53: 851-856).

[0098]

In addition to the aforementioned humanization, for example, alteration is also possible for improving the biological properties of the antibody such as binding activity against the antigen. Such alteration can be carried out by a method such as site-directed mutagenesis (see e.g., Kunkel (1985) Proc. Natl. Acad. Sci. USA 82: 488), PCR mutagenesis, or cassette mutagenesis. In general, such an antibody variant having the improved biological properties has 70% or higher, more preferably 80% or higher, further preferably 90% or higher (e.g., 95% or higher, 97%, 98%, or 99%) amino acid sequence homology and/or similarity to the variable region amino acid sequences of the original antibody. In the present specification, the sequence homology and/or similarity is defined as the percentage of amino acid residues homologous (identical amino acid residues) or similar (amino acid residues classified into the same group on the basis of the side chain properties of general amino acids) to the original antibody residues after sequence alignment and gap introduction as needed so as to attain the largest value of sequence homology. Typically, natural amino acid residues are classified on the basis of the properties of their side chains into (1) hydrophobic group: alanine, isoleucine, norleucine, valine, methionine, and leucine; (2) neutral hydrophilic group: asparagine, glutamine, cysteine, threonine, and serine; (3) acidic group: aspartic acid and glutamic acid; (4) basic group: arginine, histidine, and lysine; (5) group of residues influencing chain orientation: glycine and proline; and (6) aromatic group: tyrosine, tryptophan, and phenylalanine.

[0099]

A total of six complementarity determining regions (hypervariable domains; CDRs) present in H chain and L chain variable regions usually interact with each other to form an antigen-binding site in the antibody. Even one of these variable regions is known to have the ability to recognize and bind to the antigen, albeit with lower affinity than that of a molecule

containing the whole binding site. Thus, the genes encoding the H chain and the L chain of the antibody of the present invention can encode fragments or moieties containing the respective antigen-binding sites of the H chain and the L chain as long as the polypeptides encoded by the genes should maintain the binding activity against the desired antigen.

[0100]

Activity of polypeptide and examples of antigen

For example, an antibody or a polypeptide having an activity can be efficiently prepared by use of the method for controlling dissociation and/or association according to the present invention. Examples of the activity can include binding activity, neutralizing activity, cytotoxic activity, agonistic activity, antagonistic activity, and enzymatic activity. The agonistic activity is an activity of intracellularly transducing signals, for example, through the binding of an antibody to an antigen such as a receptor to induce change in some physiological activity. Examples of the physiological activity can include, but are not limited to, proliferative activity, survival activity, differentiation activity, transcriptional activity, membrane transport activity, binding activity, proteolytic activity, phosphorylating/dephosphorylating activity, redox activity, transfer activity, nucleolytic activity, dehydration activity, cell death-inducing activity, and apoptosis-inducing activity.

[0101]

Also, an antibody or a polypeptide that recognizes a desired antigen or binds to a desired receptor can be efficiently prepared by the method of the present invention.

[0102]

In the present specification, the antigen is not particularly limited, and any antigen can be used. Preferred examples of the antigen include ligands (cytokines, chemokines, etc.), receptors, cancer antigens, MHC antigens, differentiation antigens, immunoglobulins, and immunocomplexes partially containing an immunoglobulin.

Examples of the cytokines can include interleukins 1 to 18, colony-stimulating factors (G-CSF, M-CSF, GM-CSF, etc.), interferons (IFN-α, IFN-β, IFN-γ, etc.), growth factors (EGF, FGF, IGF, NGF, PDGF, TGF, HGF, etc.), tumor necrosis factors (TNF-α and TNF-β), lymphotoxin, erythropoietin, leptin, SCF, TPO, MCAF, and BMP.

Examples of the chemokines can include CC chemokines such as CCL1 to CCL28, CXC chemokines such as CXCL1 to CXCL17, C chemokines such as XCL1 to XCL2, and CX3C chemokines such as CX3CL1.

[0103]

Examples of the receptors can include receptors belonging to receptor families such as hematopoietic factor receptor family, cytokine receptor family, tyrosine kinase receptor family, serine/threonine kinase receptor family, TNF receptor family, G protein-coupled receptor family,

GPI-anchored receptor family, tyrosine phosphatase receptor family, adhesion factor family, and hormone receptor family. The receptors belonging to these receptor families and features thereof are described in many literatures, for example, Cooke BA., King RJB., van der Molen HJ. ed. New Comprehensive Biochemistry Vol. 18B "Hormones and their Actions Part II" pp. 1-46 (1988) Elsevier Science Publishers BV., Patthy (Cell (1990) 61 (1), 13-14), Ullrich et al. (Cell (1990) 61 (2), 203-212), Massague (e carries an acute accent) (Cell (1992) 69 (6), 1067-1070), Miyajima et al. (Annu. Rev. Immunol. (1992) 10, 295-331), Taga et al. (FASEB J. (1992) 6, 3387-3396), Fantl et al. (Annu. Rev. Biochem. (1993), 62, 453-481), Smith et al. (Cell (1994) 76 (6) 959-962), and Flower DR. (Biochim. Biophys. Acta (1999) 1422 (3) 207-234).

[0104]

Preferred examples of specific receptors belonging to the receptor families include human or mouse erythropoietin (EPO) receptor (Blood (1990) 76 (1), 31-35; and Cell (1989) 57 (2), 277-285), human or mouse granulocyte colony-stimulating factor (G-CSF) receptor (Proc. Natl. Acad. Sci. USA. (1990) 87 (22), 8702-8706; mG-CSFR; and Cell (1990) 61 (2), 341-350), human or mouse thrombopoietin (TPO) receptor (Proc Natl Acad Sci USA. (1992) 89 (12), 5640-5644; and EMBO J. (1993) 12 (7), 2645-53), human or mouse insulin receptor (Nature (1985) 313 (6005), 756-761), human or mouse Flt-3 ligand receptor (Proc. Natl. Acad. Sci. USA. (1994) 91 (2), 459-463), human or mouse platelet-derived growth factor (PDGF) receptor (Proc. Natl. Acad. Sci. USA. (1988) 85 (10) 3435-3439), human or mouse interferon (IFN)-α/β receptor (Cell (1990) 60 (2), 225-234; and Cell (1994) 77 (3), 391-400), human or mouse leptin receptor, human or mouse growth hormone (GH) receptor, human or mouse interleukin (IL)-10 receptor, human or mouse insulin-like growth factor (IGF)-I receptor, human or mouse leukemia inhibitory factor (LIF) receptor, and human or mouse ciliary neurotrophic factor (CNTF) receptor.

[0105]

The cancer antigens are antigens that are expressed with the malignant transformation of cells, and are also called tumor-specific antigens. Abnormal sugar chains that appear on cell surface or protein molecules when cells are cancerated are also included in the cancer antigens and are also called cancer carbohydrate antigens. Preferred examples of the cancer antigens include GPC3 that belongs to the GPI-anchored receptor family as the aforementioned receptors but is expressed in some cancers including liver cancer (Int J Cancer. (2003) 103 (4), 455-65), EpCAM that is expressed in a plurality of cancers including lung cancer (Proc Natl Acad Sci USA. (1989) 86 (1), 27-31), CA19-9, CA15-3, and sialyl SSEA-1 (SLX).

[0106]

The MHC antigens are mainly classified into MHC class I antigens and MHC class II antigens. The MHC class I antigens include HLA-A, -B, -C, -E, -F, -G, and -H. The MHC class II antigens include HLA-DR, -DQ, and -DP.

[0107]

The differentiation antigens can include CD1, CD2, CD4, CD5, CD6, CD7, CD8, CD10, CD11a, CD11b, CD11c, CD13, CD14, CD15s, CD16, CD18, CD19, CD20, CD21, CD23, CD25, CD28, CD29, CD30, CD32, CD33, CD34, CD35, CD38, CD40, CD41a, CD41b, CD42a, CD42b, CD43, CD44, CD45, CD45RO, CD48, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD51, CD54, CD55, CD56, CD57, CD58, CD61, CD62E, CD62L, CD62P, CD64, CD69, CD71, CD73, CD95, CD102, CD106, CD122, CD126, and CDw130.

[0108]

The immunoglobulins include IgA, IgM, IgD, IgG, and IgE. The immunocomplexes contain at least any component of immunoglobulins.

Other examples of the antigen can include the following molecules: 17-IA, 4-1BB, 4Dc, 6-keto-PGF1a, 8-iso-PGF2a, 8-oxo-dG, A1 adenosine receptor, A33, ACE, ACE-2, activin, activin A, activin AB, activin B, activin C, activin RIA, activin RIA ALK-2, activin RIB ALK-4, activin RIIA, activin RIIB, ADAM, ADAM10, ADAM12, ADAM15, ADAM17/TACE, ADAM8, ADAM9, ADAMTS, ADAMTS4, ADAMTS5, addressin, aFGF, ALCAM, ALK, ALK-1, ALK-7, alpha-1-antitrypsin, alpha-V/beta-1 antagonist, ANG, Ang, APAF-1, APE, APJ, APP, APRIL, AR, ARC, ART, artemin, anti-Id, ASPARTIC, atrial natriuretic factor, av/b3 integrin, Axl, b2M, B7-1, B7-2, B7-H, B-lymphocyte-stimulating factor (BlyS), BACE, BACE-1, Bad, BAFF, BAFF-R, Bag-1, BAK, Bax, BCA-1, BCAM, Bcl, BCMA, BDNF, b-ECGF, bFGF, BID, Bik, BIM, BLC, BL-CAM, BLK, BMP, BMP-2 BMP-2a, BMP-3 (osteogenin), BMP-4 BMP-2b, BMP-5, BMP-6 Vgr-1, BMP-7 (OP-1), BMP-8 (BMP-8a, OP-2), BMPR, BMPR-IA (ALK-3), BMPR-IB (ALK-6), BRK-2, RPK-1, BMPR-II (BRK-3), BMP, b-NGF, BOK, bombesin, bone-derived neurotrophic factor, BPDE, BPDE-DNA, BTC, complement factor 3 (C3), C3a, C4, C10, CA125, CAD-8, calcitonin, cAMP, carcinoembryonic antigen (CEA), cancer-associated antigen, cathepsin A, cathepsin B, cathepsin C/DPPI, cathepsin D, cathepsin E, cathepsin H, cathepsin L, cathepsin O, cathepsin S, cathepsin V, cathepsin X/Z/P, CBL, CCI, CCK2, CCL, CCL1, CCL11, CCL12, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL2, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, CCL28, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9/10, CCR, CCR1, CCR10, CCR10, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CD1, CD2, CD3, CD3E, CD4, CD5, CD6, CD7, CD8, CD10, CD11a, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD21, CD22, CD23, CD25, CD27L, CD28, CD29, CD30, CD30L, CD32, CD33 (p67 protein), CD34, CD38, CD40, CD40L, CD44, CD45, CD46, CD49a, CD52, CD54, CD55, CD56, CD61, CD64,

CD66e, CD74, CD80 (B7-1), CD89, CD95, CD123, CD137, CD138, CD140a, CD146, CD147, CD148, CD152, CD164, CEACAM5, CFTR, cGMP, CINC, *Clostridium botulinum* toxin, *Clostridium perfringens* toxin, CKb8-1, CLC, CMV, CMV UL, CNTF, CNTN-1, COX, C-Ret, CRG-2, CT-1, CTACK, CTGF, CTLA-4, CX3CL1, CX3CR1, CXCL, CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CXCL15, CXCL16, CXCR, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, cytokeratin tumor-associated antigen, DAN, DCC, DcR3, DC-SIGN, decay accelerating factor, des(1-3)-IGF-I (brain IGF-1), Dhh, digoxin, DNAM-1, Dnase, Dpp, DPPIV/CD26, Dtk, ECAD, EDA, EDA-A1, EDA-A2, EDAR, EGF, EGFR (ErbB-1), EMA, EMMPRIN, ENA, endothelin receptor, enkephalinase, eNOS, Eot, eotaxin 1, EpCAM, ephrin B2/EphB4, EPO, ERCC, E-selectin, ET-1, factor IIa, factor VII, factor VIIIc, factor IX, fibroblast-activating protein (FAP), Fas, FcR1, FEN-1, ferritin, FGF, FGF-19, FGF-2, FGF3, FGF-8, FGFR, FGFR-3, fibrin, FL, FLIP, Flt-3, Flt-4, follicle-stimulating hormone, fractalkine, FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, FZD10, G250, Gas6, GCP-2, GCSF, GD2, GD3, GDF, GDF-1, GDF-3 (Vgr-2), GDF-5 (BMP-14, CDMP-1), GDF-6 (BMP-13, CDMP-2), GDF-7 (BMP-12, CDMP-3), GDF-8 (myostatin), GDF-9, GDF-15 (MIC-1), GDNF, GDNF, GFAP, GFRa-1, GFR-alpha 1, GFR-alpha 2, GFR-alpha 3, GITR, glucagon, Glut4, glycoprotein IIb/IIIa (GPIIb/IIIa), GM-CSF, gp130, gp72, GRO, growth hormone-releasing factor, hapten (NP-cap or NIP-cap), HB-EGF, HCC, HCMV gB envelope glycoprotein, HCMV gH envelope glycoprotein, HCMV UL, hematopoietic growth factor (HGF), Hep B gp120, heparanase, Her2, Her2/neu (ErbB-2), Her3 (ErbB-3), Her4 (ErbB-4), herpes simplex virus (HSV) gB glycoprotein, HSV gD glycoprotein, HGFA, high-molecular-weight melanoma-associated antigen (HMW-MAA), HIV gp120, HIV IIIB gp 120 V3 loop, HLA, HLA-DR, HM1.24, HMFG PEM, HRG, Hrk, human heart myosin, human cytomegalovirus (HCMV), human growth hormone (HGH), HVEM, I-309, IAP, ICAM, ICAM-1, ICAM-3, ICE, ICOS, IFNg, Ig, IgA receptor, IgE, IGF, IGF-binding protein, IGF-1R, IGFBP, IGF-I, IGF-II, IL, IL-1, IL-1R, IL-2, IL-2R, IL-4, IL-4R, IL-5, IL-5R, IL-6, IL-6R, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-18, IL-18R, IL-23, interferon (INF)-alpha, INF-beta, INF-gamma, inhibin, iNOS, insulin A chain, insulin B chain, insulin-like growth factor 1, integrin alpha 2, integrin alpha 3, integrin alpha 4, integrin alpha 4/beta 1, integrin alpha 4/beta 7, integrin alpha 5 (alpha V), integrin alpha 5/beta 1, integrin alpha 5/beta 3, integrin alpha 6, integrin beta 1, integrin beta 2, interferon gamma, IP-10, I-TAC, JE, kallikrein 2, kallikrein 5, kallikrein 6, kallikrein 11, kallikrein 12, kallikrein 14, kallikrein 15, kallikrein L1, kallikrein L2, kallikrein L3, kallikrein L4, KC, KDR, keratinocyte growth factor (KGF), laminin 5, LAMP, LAP, LAP (TGF-1), latent TGF-1, latent TGF-1 bp1, LBP, LDGF, LECT2, lefty, Lewis-Y antigen, Lewis-Y-related antigen, LFA-1, LFA-3, Lfo, LIF, LIGHT, lipoprotein, LIX, LKN, Lptn, L-selectin, LT-a, LT-b, LTB4, LTBP-1, lung surfactant, luteinizing hormone,

lymphotoxin beta receptor, Mac-1, MAdCAM, MAG, MAP2, MARC, MCAM, MCAM, MCK-2, MCP, M-CSF, MDC, Mer, metalloprotease, MGDF receptor, MGMT, MHC (HLA-DR), MIF, MIG, MIP, MIP-1-alpha, MK, MMAC1, MMP, MMP-1, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-15, MMP-2, MMP-24, MMP-3, MMP-7, MMP-8, MMP-9, MPIF, Mpo, MSK, MSP, mucin (Muc1), MUC18, mullerian-inhibiting factor, Mug, MuSK, NAIP, NAP, NCAD, N-cadherin, NCA 90, NCAM, NCAM, neprilysin, neurotrophin-3, -4, or -6, neurturin, nerve growth factor (NGF), NGFR, NGF-beta, nNOS, NO, NOS, Npn, NRG-3, NT, NTN, OB, OGG1, OPG, OPN, OSM, OX40L, OX40R, p150, p95, PADPr, parathyroid hormone, PARC, PARP, PBR, PBSF, PCAD, P-cadherin, PCNA, PDGF, PDGF, PDK-1, PECAM, PEM, PF4, PGE, PGF, PGI2, PGJ2, PIN, PLA2, placental alkaline phosphatase (PLAP), PlGF, PLP, PP14, proinsulin, prorelaxin, protein C, PS, PSA, PSCA, prostate-specific membrane antigen (PSMA), PTEN, PTHrp, Ptk, PTN, R51, RANK, RANKL, RANTES, RANTES, relaxin A chain, relaxin B chain, renin, respiratory syncytial virus (RSV) F, RSV Fgp, Ret, rheumatoid factor, RLIP76, RPA2, RSK, S100, SCF/KL, SDF-1, SERINE, serum albumin, sFRP-3, Shh, SIGIRR, SK-1, SLAM, SLPI, SMAC, SMDF, SMOH, SOD, SPARC, Stat, STEAP, STEAP-II, TACE, TACI, TAG-72 (tumor-associated glycoprotein-72), TARC, TCA-3, T cell receptor (e.g., T cell receptor alpha/beta), TdT, TECK, TEM1, TEM5, TEM7, TEM8, TERT, testicular PLAP-like alkaline phosphatase, TfR, TGF, TGF-alpha, TGF-beta, TGF-beta Pan Specific, TGF-beta RI (ALK-5), TGF-beta RII, TGF-beta RIIb, TGF-beta RIII, TGF-beta 1, TGF-beta 2, TGF-beta 3, TGF-beta 4, TGF-beta 5, thrombin, thymus Ck-1, thyroid stimulating hormone, Tie, TIMP, TIQ, tissue factor, TMEFF2, Tmpo, TMPRSS2, TNF, TNF-alpha, TNF-alpha/beta, TNF-beta 2, TNFc, TNF-RI, TNF-RII, TNFRSF10A (TRAIL R1 Apo-2, DR4), TNFRSF10B (TRAIL R2 DR5, KILLER, TRICK-2A, TRICK-B), TNFRSF10C (TRAIL R3 DcR1, LIT, TRID), TNFRSF10D (TRAIL R4 DcR2, TRUNDD), TNFRSF11A (RANK ODF R, TRANCE R), TNFRSF11B (OPG OCIF, TR1), TNFRSF12 (TWEAK R FN14), TNFRSF13B (TACI), TNFRSF13C (BAFF R), TNFRSF14 (HVEM ATAR, HveA, LIGHT R, TR2), TNFRSF16 (NGFR p75NTR), TNFRSF17 (BCMA), TNFRSF18 (GITR AITR), TNFRSF19 (TROY TAJ, TRADE), TNFRSF19L (RELT), TNFRSF1A (TNF RI CD120a, p55-60), TNFRSF1B (TNF RII CD120b, p75-80), TNFRSF26 (TNFRH3), TNFRSF3 (LTbR TNF RIII, TNFC R), TNFRSF4 (OX40 ACT35, TXGP1 R), TNFRSF5 (CD40 p50), TNFRSF6 (Fas Apo-1, APT1, CD95), TNFRSF6B (DcR3 M68, TR6), TNFRSF7 (CD27), TNFRSF8 (CD30), TNFRSF9 (4-1BB CD137, ILA), TNFRSF21 (DR6), TNFRSF22 (DcTRAIL R2 TNFRH2), TNFRST23 (DcTRAIL R1 TNFRH1), TNFRSF25 (DR3 Apo-3, LARD, TR-3, TRAMP, WSL-1), TNFSF10 (TRAIL Apo-2 ligand, TL2), TNFSF11 (TRANCE/RANK ligand ODF, OPG ligand), TNFSF12 (TWEAK Apo-3 ligand, DR3 ligand), TNFSF13 (APRIL TALL2), TNFSF13B (BAFF BLYS, TALL1, THANK, TNFSF20), TNFSF14 (LIGHT HVEM ligand, LTg), TNFSF15 (TL1A/VEGI), TNFSF18 (GITR ligand

AITR ligand, TL6), TNFSF1A (TNF-a conectin, DIF, TNFSF2), TNFSF1B (TNF-b LTa, TNFSF1), TNFSF3 (LTb TNFC, p33), TNFSF4 (OX40 ligand gp34, TXGP1), TNFSF5 (CD40 ligand CD154, gp39, HIGM1, IMD3, TRAP), TNFSF6 (Fas ligand Apo-1 ligand, APT1 ligand), TNFSF7 (CD27 ligand CD70), TNFSF8 (CD30 ligand CD153), TNFSF9 (4-1BB ligand CD137 ligand), TP-1, t-PA, Tpo, TRAIL, TRAIL R, TRAIL-R1, TRAIL-R2, TRANCE, transferrin receptor, TRF, Trk, TROP-2, TSG, TSLP, tumor-associated antigen CA125, tumor-associated antigen exhibiting Lewis Y-related carbohydrate, TWEAK, TXB2, Ung, uPAR, uPAR-1, urokinase, VCAM, VCAM-1, VECAD, VE-cadherin, VE-cadherin-2, VEFGR-1 (flt-1), VEGF, VEGFR, VEGFR-3 (flt-4), VEGI, VIM, viral antigen, VLA, VLA-1, VLA-4, VNR integrin, von Willebrand factor, WIF-1, WNT1, WNT2, WNT2B/13, WNT3, WNT3A, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT9A, WNT9A, WNT9B, WNT10A, WNT10B, WNT11, WNT16, XCL1, XCL2, XCR1, XCR1, XEDAR, XIAP, XPD, HMGB1, IgA, Aβ, CD81, CD97, CD98, DDR1, DKK1, EREG, Hsp90, IL-17/IL-17R, IL-20/IL-20R, oxidized LDL, PCSK9, prekallikrein, RON, TMEM16F, SOD1, Chromogranin A, Chromogranin B, tau, VAP1, high-molecular-weight kininogen, IL-31, IL-31R, Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.5, Nav1.6, Nav1.7, Nav1.8, Nav1.9, EPCR, C1, C1q, C1r, C1s, C2, C2a, C2b, C3, C3a, C3b, C4, C4a, C4b, C5, C5a, C5b, C6, C7, C8, C9, factor B, factor D, factor H, properdin, sclerostin, fibrinogen, fibrin, prothrombin, thrombin, tissue factor, factor V, factor Va, factor VII, factor VIIa, factor VIII, factor VIIIa, factor IX, factor IXa, factor X, factor Xa, factor XI, factor XIa, factor XII, factor XIIa, factor XIII, factor XIIIa, TFPI, antithrombin III, EPCR, thrombomodulin, TAPI, tPA, plasminogen, plasmin, PAI-1, PAI-2, GPC3, Syndecan-1, Syndecan-2, Syndecan-3, Syndecan-4, LPA, S1P, and receptors for hormones and growth factors.

[0109]

In a non-limiting embodiment of the present invention, one specificity of the bispecific antibody can target a cancer antigen, and the other specificity can target an antigen expressed on CTL (cytotoxic T lymphocyte), for example, CD3 or TNFRSF (tumor necrosis factor receptor super family), though these specificities are not limited to this combination. Examples of the TNFRSF include TNFRSF9 (CD137), TNFRSF5 (CD40), and TNFRSF4 (OX40).

[0110]

Alteration of nucleic acid

In another aspect of the production method of the present invention, the present invention provides a method for producing a heteromultimer having a mutation in amino acid residues that form the interface between polypeptides (e.g., amino acid residues at EU numbering positions 356 and 439, positions 357 and 370, and positions 399 and 409), and/or an amino acid residue at EU numbering position 397 and/or 392 so as to control dissociation and/or association between the polypeptides, the production method comprising the steps of: (a) altering nucleic acids

encoding the amino acid residues that form the interface between polypeptides, etc., from their original nucleic acids so as to control dissociation and association between the polypeptides; (b) culturing a host cell having the nucleic acids to express the polypeptides; (c) recovering the polypeptides from the cultures of the host cell; and (d) incubating these polypeptides under a reducing condition to recover a heteromer of the desired polypeptides.

[0111]

In a preferred embodiment, the production method of the present invention is also a method comprising the step of altering nucleic acids encoding the amino acid residues that form the interface between polypeptides from their original nucleic acids by use of the aforementioned method for controlling dissociation and/or association according to the present invention so as to inhibit association between the polypeptides.

[0112]

In the method of the present invention, the phrase "altering nucleic acids" means to alter nucleic acids so as to correspond to the amino acid residues that are introduced by the "alteration" according to the present invention. More specifically, the phrase "altering nucleic acids" means to alter nucleic acids encoding the original amino acid residues (amino acid residues before the alteration) to nucleic acids encoding the amino acid residues that are introduced by the alteration. Usually, this phrase means to carry out gene manipulation or mutation treatment for the insertion, deletion, or substitution of at least one base in the original nucleic acids so as to become codons encoding the amino acid residues of interest. Specifically, the codons encoding the original amino acid residues are substituted by codons encoding the amino acid residues that are introduced by the alteration. Such nucleic acid alteration can be appropriately carried out using a technique generally known to those skilled in the art, for example, site-directed mutagenesis or PCR mutagenesis.

[0113]

The nucleic acids according to the present invention are usually carried by (or inserted in) appropriate vectors and transferred to host cells. The vectors are not particularly limited as long as the vectors can stably retain the inserted nucleic acids. For example, when *E. coli* is used as the host, pBluescript vectors (manufactured by Stratagene Corp.) or the like are preferred as vectors for cloning. Various commercially available vectors can be used. In the case of using the vectors for the purpose of producing the polypeptide of the present invention, expression vectors are particularly useful. The expression vectors are not particularly limited as long as the vectors permit expression of the polypeptide *in vitro*, in *E. coli*, in cultured cells, or in organism individuals. The expression vectors are preferably, for example, pBEST vectors (manufactured by Promega K.K.) for *in vitro* expression, pET vectors (manufactured by Invitrogen Corp.) for *E. coli*, pME18S-FL3 vectors (GenBank Accession No. AB009864) for

cultured cells, and pME18S vectors (Mol Cell Biol. 8: 466-472 (1988)) for organism individuals. The insertion of the DNAs of the present invention into the vectors can be carried out by a routine method, for example, ligase reaction using restriction sites (Current protocols in Molecular Biology edit. Ausubel et al. (1987) Publish. John Wiley & Sons. Section 11.4-11.11).

[0114]

The host cells are not particularly limited, and various host cells are used according to the purpose. Examples of the cells for polypeptide expression can include bacterial cells (e.g., *Streptococcus*, *Staphylococcus*, *E. coli*, *Streptomyces*, and *Bacillus subtilis*), fungus cells (e.g., yeasts and *Aspergillus*), insect cells (e.g., *Drosophila* S2 and *Spodoptera* SF9), animal cells (e.g., CHO, COS, HeLa, C127, 3T3, BHK, HEK293, and Bowes melanoma cells), and plant cells. The transfer of the vectors to the host cells can be carried out by a method known in the art, for example, a calcium phosphate precipitation method, an electroporation method (Current protocols in Molecular Biology edit. Ausubel et al., (1987) Publish. John Wiley & Sons. Section 9.1-9.9), a Lipofectamine method (manufactured by GIBCO-BRL/Life Technologies, Inc.), or a microinjection method.

[0115]

An appropriate secretory signal can be incorporated into the polypeptide of interest in order to secrete the polypeptide expressed in the host cells to the lumen of the endoplasmic reticulum, periplasmic space, or an extracellular environment. The signal may be endogenous to the polypeptide of interest or may be a foreign signal.

[0116]

When the polypeptide of the present invention is secreted into a medium, the recovery of the polypeptide in the production method is carried out by the recovery of the medium. When the polypeptide of the present invention is produced into cells, the cells are first lysed and then the polypeptide is recovered.

[0117]

A method known in the art including ammonium sulfate or ethanol precipitation, acid extraction, anion- or cation-exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography, and lectin chromatography can be used for recovering and purifying the polypeptide of the present invention from the recombinant cell cultures.

[0118]

In a non-limiting embodiment of the present invention, examples of the production method include: a method which involves separately culturing cell lines respectively producing the homo variants of the first and second polypeptides, and purifying the culture supernatants, followed by FAE (Fab arm exchange) reaction using the purified antibodies; a method which

involves separately culturing cell lines respectively producing the homo variants of the first and second polypeptides, mixing the culture supernatants without purification, and causing FAE reaction in the mixed culture supernatant, followed by purification; a method which involves mixing a cell line producing the homo variant of the first polypeptides with a cell line producing the homo variant of the second polypeptides, culturing the mixture, and purifying the culture supernatant, followed by FAE reaction using the purified antibodies; and a method which involves mixing a cell line producing the homo variant of the first polypeptides with a cell line producing the homo variant of the second polypeptides, culturing the mixture, and causing FAE reaction in the culture supernatant, followed by purification.

[0119]

In a non-limiting embodiment, the present invention provides a method for producing a heteromultimer, comprising the following steps a) to c):

a) mixing a cell line producing the homo variant of the first polypeptides with a cell line producing the homo variant of the second polypeptides;

b) incubating the homo variant of the first polypeptides and the homo variant of the second polypeptides together so as to allow cysteines in hinge regions to cause disulfide bond isomerization in the culture supernatant; and

c) obtaining a heteromultimer comprising the first and second polypeptides.

[0120]

In a non-limiting embodiment, the present invention provides a method for producing a heteromultimer, comprising the following steps a) to c):

a) separately culturing cell lines respectively producing the homo variants of the first and second polypeptides;

b) mixing the respective culture supernatants of the cell lines and incubating the homo variant of the first polypeptides and the homo variant of the second polypeptides together so as to allow cysteines in hinge regions to cause disulfide bond isomerization; and

c) obtaining a heteromultimer comprising the first and second polypeptides.

[0121]

<u>Method for selecting desired heteromultimer</u>

The present invention further provides a method for selecting a desired heteromultimer. In a preferred embodiment, the method is a method for selecting a heteromultimer having desired properties, comprising the following steps:

a) providing a first polypeptide set and a second polypeptide set, wherein each polypeptide constituting the first set has target specificity different from that of each polypeptide constituting the second set, and each polypeptide constituting the first and second sets contains the amino

acid alteration related to interface control using charge repulsion and/or the amino acid alteration to destabilize the stability of a CH3 region;

b) incubating each polypeptide constituting the first set together with each polypeptide constituting the second set under a reducing condition, thereby preparing a mixture of plural types of heteromultimers;

c) assaying the resulting mixture of plural types of heteromultimers for the predetermined desired properties; and

d) selecting a heteromultimer having the desired properties.

[0122]

Pharmaceutical composition

The present invention also relates to a composition (drug) comprising the heteromultimer of the present invention and a pharmaceutically acceptable carrier.

[0123]

In the present invention, the pharmaceutical composition usually refers to a drug for the treatment or prevention of a disease or for testing or diagnosis.

[0124]

The pharmaceutical composition of the present invention can be formulated by a method generally known to those skilled in the art. For example, the pharmaceutical composition can be used in the form of a parenteral injection of an aseptic solution or suspension with water or any other pharmaceutically acceptable solution. For example, the pharmaceutical composition may be formulated with the heteromultimer mixed in a unit dosage form required for generally accepted pharmaceutical practice, in appropriate combination with pharmacologically acceptable carriers or media, specifically, sterilized water, physiological saline, plant oil, an emulsifier, a suspending agent, a surfactant, a stabilizer, a flavoring agent, an excipient, a vehicle, a preservative, a binder, etc. The amount of the active ingredient in these preparations is set so as to give an appropriate volume within a prescribed range.

[0125]

An aseptic composition for injection can be formulated according to conventional pharmaceutical practice using a vehicle such as injectable distilled water.

[0126]

Examples of aqueous solutions for injection include physiological saline, and isotonic solutions containing glucose and other adjuvants (e.g., D-sorbitol, D-mannose, D-mannitol, and sodium chloride). These solutions may be used in combination with an appropriate solubilizer, for example, an alcohol (ethanol, etc.) or a polyalcohol (propylene glycol, polyethylene glycol, etc.), or a nonionic surfactant (polysorbate 80(TM), HCO-50, etc.).

[0127]

Examples of oily solutions include sesame oil and soybean oil. These solutions may be used in combination with benzyl benzoate and/or benzyl alcohol as a solubilizer. The solutions may be further mixed with a buffer (e.g., a phosphate buffer solution and a sodium acetate buffer solution), a soothing agent (e.g., procaine hydrochloride), a stabilizer (e.g., benzyl alcohol and phenol), and an antioxidant. The injection solutions thus prepared are usually charged into appropriate ampules.

[0128]

The pharmaceutical composition of the present invention is preferably administered parenterally. The composition can be in the dosage form of, for example, an injection, a nasal administration agent, a transpulmonary administration agent, or a percutaneous administration agent. The pharmaceutical composition can be administered systemically or locally through, for example, intravenous injection, intramuscular injection, intraperitoneal injection, or subcutaneous injection.

[0129]

The administration method can be appropriately selected depending on the age and symptoms of a patient. The dose of a pharmaceutical composition containing an antibody or a polynucleotide encoding the antibody can be set to within a range of, for example, 0.0001 to 1000 mg/kg of body weight per dose. Alternatively, the dose may be, for example, 0.001 to 100000 mg per patient, though the present invention is not necessarily limited by these numeric values. Although the dose and the administration method vary depending on the weight, age, symptoms, etc., of a patient, those skilled in the art can appropriately select an appropriate dose and administration method in consideration of their conditions.

[0130]

In the present invention, the heteromultimer of the present invention is useful as an active ingredient for a therapeutic or preventive agent for a cancer. Examples of the cancer include, but are not limited to: lung cancer (including small-cell lung cancer, non-small-cell lung cancer, lung adenocarcinoma, and lung squamous cell carcinoma), large bowel cancer, rectal cancer, colon cancer, breast cancer, liver cancer, stomach cancer, pancreatic cancer, kidney cancer, prostate cancer, ovary cancer, thyroid gland cancer, bile duct cancer, peritoneal cancer, mesothelioma, squamous cell cancer, uterine cervix cancer, uterine body cancer, bladder cancer, esophagus cancer, head and neck cancer, nasopharyngeal cancer, salivary gland tumor, thymoma, skin cancer, basal cell tumor, malignant melanoma, anus cancer, penis cancer, testis cancer, Wilms's tumor, acute myeloid leukemia (including acute myeloleukemia, acute myeloblastic leukemia, acute promyelocytic leukemia, acute myelomonocytic leukemia, and acute monocytic leukemia), chronic myeloid leukemia, acute lymphoid leukemia, chronic lymphoid leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma (Burkitt's lymphoma, chronic lymphocytic

leukemia, mycosis fungoides, mantle cell lymphoma, follicular lymphoma, diffuse large-cell lymphoma, marginal zone lymphoma, hairy cell leukemia, plasmacytoma, peripheral T-cell lymphoma, and adult T-cell leukemia/lymphoma), Langerhans' cell histiocytosis, multiple myeloma, myelodysplastic syndrome, brain tumor (including glioma, astroglioma, glioblastoma, meningioma, and ependymoma), neuroblastoma, retinoblastoma, osteosarcoma, Kaposi's sarcoma, Ewing's sarcoma, angiosarcoma, and hemangiopericytoma.

[0131]

If necessary, the polypeptide or the heteromultimer of the present invention can be made into preparations in combination with other pharmaceutical ingredients.

[0132]

The present invention also provides a kit for use in the treatment method or prevention method of the present invention, comprising at least a heteromultimer produced by the production method of the present invention or the pharmaceutical composition of the present invention. In the kit, for example, a pharmaceutically acceptable carrier, a vehicle, or an instruction stating the usage can also be additionally packaged. The present invention also relates to use of the polypeptide of the present invention or a polypeptide produced by the production method of the present invention for producing a therapeutic or preventive agent for immunological and inflammatory diseases. The present invention further relates to the polypeptide of the present invention or a polypeptide produced by the production method of the present invention for use in the treatment method or the prevention method of the present invention.

[0133]

The three-letter codes of the amino acids used herein and their corresponding one-letter codes are as follows:

Alanine: Ala: A
Arginine: Arg: R
Asparagine: Asn: N
Aspartic acid: Asp: D
Cysteine: Cys: C
Glutamine: Gln: Q
Glutamic acid: Glu: E
Glycine: Gly: G
Histidine: His: H
Isoleucine: Ile: I
Leucine: Leu: L
Lysine: Lys: K

Methionine: Met: M
Phenylalanine: Phe: F
Proline: Pro: P
Serine: Ser: S
Threonine: Thr: T
Tryptophan: Trp: W
Tyrosine: Tyr: Y
Valine: Val: V

[0134]

All prior technical literatures cited herein are incorporated herein by reference.

Examples

[0135]

[Example 1] Study on improvement in Fab arm exchange efficiency by introduction of association interface-controlling alteration to antibody

In Fab arm exchange, two types of homomeric antibodies are mixed in the presence of a reducing agent, and the resulting four H-L chain pairs of the antibody molecules (referred to as half-molecules or HL molecules, each of which is a molecule composed of one heavy chain and one light chain) reassociate by swapping to yield bispecific antibodies. Since the reassociation of HL molecules occurs at random, the bispecific antibody of interest is theoretically obtained at only 50% of the total amount of antibodies present in the system. Provided that different charges are introduced in advance to two types of homomeric antibodies, it is assumed that heterodimerization can occur preferentially over homodimerization during the reassociation of the resulting HL molecules to prepare a bispecific antibody with high efficiency. Accordingly, the alteration to control association interface between antibody CH3 regions (alteration to promotion of hetero-association of two types of H chains through the use of the charge interaction and repulsion between their CH3 regions) as reported in WO2006/106905 was used to test whether or not it can improve the reaction efficiency of Fab arm exchange (rate of bispecific antibody formation).

[0136]

The antibody H chain variable regions used were H chain variable regions WT(H) (SEQ ID NO: 1; hereinafter, referred to as MRAH) and H54 (SEQ ID NO: 2) of the antibody against human interleukin 6 receptor disclosed in WO2009/125825. MRAH-G1d (SEQ ID NO: 3) and H54-G1d (SEQ ID NO: 4) having an antibody H chain constant region G1d derived from a human IgG1 H chain constant region by the removal of C-terminal Gly and Lys and MRAH-wtG4d (SEQ ID NO: 5) and H54-wtG4d (SEQ ID NO: 6) having an antibody H chain constant

region wtG4d derived from a human IgG4 H chain constant region by the removal of C-terminal Gly and Lys were prepared using the H chain variable regions. Next, P228S and K409R alterations were introduced to MRAH-G1d and H54-G1d to prepare MRAH-G1dsr (SEQ ID NO: 7) and H54-G1dsr (SEQ ID NO: 8) having an IgG4-type hinge sequence and CH3 domain sequence. D356K was further introduced as association interface-controlling alteration to MRAH-G1dsr to prepare MRAH-G1dsrP1 (SEQ ID NO: 9). K439E was further introduced as association interface-controlling alteration to H54-G1dsr to prepare H54-G1dsrN1 (SEQ ID NO: 10). E356K was further introduced as association interface-controlling alteration to MRAH-wtG4d to prepare MRAH-wtG4dP1 (SEQ ID NO: 11). K439E was further introduced as association interface-controlling alteration to H54-wtG4d to prepare H54-wtG4dN1 (SEQ ID NO: 12). Antibody L chains MRAL-k0 (SEQ ID NO: 13) and L28-k0 (SEQ ID NO: 14) were used for the H chain variable regions MRAH and H54, respectively. MRAH-G1dsr/MRAL-k0, H54-G1dsr/L28-k0, MRAH-G1dsrP1/MRAL-k0, H54-G1dsrN1/L28-k0, MRAH-wtG4d/MRAL-k0, H54-wtG4d/L28-k0, MRAH-wtG4dP1/MRAL-k0, and H54-wtG4dN1/L28-k0 were expressed and purified according to the method of Reference Example 1.

[0137]

Next, two types of homo variants thus obtained were mixed in the combinations given below, and the reaction products were evaluated according to the method of Reference Example 2.

(1) MRAH-wtG4d/MRAL-k0 and H54-wtG4d/L28-k0
(2) MRAH-wtG4dP1/MRAL-k0 and H54-wtG4dN1/L28-k0
(3) MRAH-G1dsr/MRAL-k0 and H54-G1dsr/L28-k0
(4) MRAH-G1dsrP1/MRAL-k0 and H54-G1dsrN1/L28-k0

Reaction conditions: in PBS (Sigma-Aldrich Corp., pH 7.4), [each mAb] = 0.2 mg/ml, [GSH (Sigma-Aldrich Corp.)] = 0.5 mM, 0.05% Tween 20 (Junsei Chemical Co., Ltd.), 37°C, 24 hours.

[0138]

The two types of antibody variable regions MRAH/MRAL and H54/L28 used in this study differ largely in pI. Therefore, peaks corresponding to their respective homo variants and the resulting bispecific antibodies can be easily separated by ion-exchange chromatography, and the reaction efficiency can be evaluated. Figure 1 shows the results of evaluating the reaction products by ion-exchange chromatography. The reaction product wtG4d produced from MRAH-wtG4d/MRAL-k0 and H54-wtG4d/L28-k0 and the reaction product G1dsr produced from MRAH-G1dsr/MRAL-k0 and H54-G1dsr/L28-k0 carrying no association interface-controlling alteration had 50.5% and 52.7% rates, respectively, of bispecific antibody formation. By contrast, the reaction product wtG4dP1/N1 produced from MRAH-wtG4dP1/MRAL-k0 and

H54-wtG4dN1/L28-k0 carrying the association interface-controlling alteration had 99.0% rate of bispecific antibody formation, and the reaction product G1dsrP1/N1 produced from MRAH-G1dsrP1/MRAL-k0 and H54-G1dsrN1/L28-k0 carrying the association interface-controlling alteration had 98.5% rate of bispecific antibody formation. Thus, the bispecific antibody was found to be formed with exceedingly high efficiency. These results demonstrated that the bispecific antibody can be prepared with exceedingly high efficiency by mixing two types of homo variants carrying the association interface-controlling alteration reported in WO2006/106905 in the presence of a reducing agent.

[0139]

[Example 2] Fab arm exchange in homo variant having hinge sequence of human naturally occurring IgG1

In Example 1, Fab arm exchange was conducted by the introduction of P228S alteration to IgG1 in order to obtain a hinge region having a sequence of naturally occurring human IgG4 type. However, naturally occurring IgG4 administered into a living body reportedly causes half-molecule exchange with endogenous IgG4. This is due to Ser at EU numbering position 228 in the hinge region. The substitution of this amino acid by IgG1-type Pro has been reported to improve stability and to prevent the *in vivo* exchange (Labrijn AF et al., Nat. Biotechnol. 2009, 27, 767-771). Thus, in consideration of administration into a living body, the hinge sequence of the prepared bispecific antibody is desirably 226C-227P-228P-229C. Accordingly, this study was conducted to test whether or not to efficiently cause Fab arm exchange by the introduction of association interface-controlling alteration even using the hinge sequence of naturally occurring human IgG1.

[0140]

First, K409R and D356K were introduced to MRAH-G1d to prepare MRAH-G1drP1 (SEQ ID NO: 15), and K409R and K439E were introduced to H54-G1d to prepare H54-G1drN1 (SEQ ID NO: 16). Antibody L chains MRAL-k0 and L28-k0 were used for the H chain variable regions MRAH and H54, respectively. MRAH-G1drP1/MRAL-k0 and H54-G1drN1/L28-k0 were expressed and purified according to the method of Reference Example 1. Next, two types of homo variants thus obtained were mixed under reaction conditions given below, and the reaction products were evaluated according to the method of Reference Example 2.

Reaction condition: in TBS (Takara Bio Inc., pH 7.6), [each mAb] = 0.2 mg/ml, 0.05% Tween 20 (Junsei Chemical Co., Ltd.), 37°C, 24 hours. The study was conducted under 3 conditions of a reducing agent [GSH (Sigma-Aldrich Corp.)] = 0.5 mM or 5 mM or [2-MEA (Sigma-Aldrich Corp.)] = 25 mM.

[0141]

Figure 2 shows the results of analyzing the reaction products according to the method of Reference Example 2. The rate of bispecific antibody formation under the same condition as in Example 1 (GSH = 0.5 mM) was 21.8%, which was drastically reduced compared with the efficiency of the case where the amino acid residue at EU numbering position 228 was Ser. By contrast, the rate of bispecific antibody formation under the reducing condition of 2-MEA (25 mM) or GSH (5 mM) was 99% or more. These results demonstrated that the bispecific antibody can be prepared with high efficiency by introducing association interface-controlling alteration and using an appropriate reducing condition even if the hinge sequence is the sequence of naturally occurring human IgG1.

[0142]

[Example 3] Fab arm exchange using CH3 of human naturally occurring IgG1

The preceding studies showed that the bispecific antibody of interest is obtained with exceedingly high efficiency by Fab arm exchange by introducing K409R alteration (which gives IgG4-type CH3) to human IgG1 and association interface-controlling alteration (D356K and K439E).

Meanwhile, it is known that if an amino acid residue at position 409 is Arg, the stability of the antibody is reduced under an acidic condition (WO/2009/041613). Antibody drug production inevitably requires a virus inactivation step of exposing the antibody under an acidic condition. In this respect, the stability of the antibody under an acidic condition is desirably high for keeping the quality of the antibody. Accordingly, it is desirable that the amino acid residue at position 409 should not be Arg. On the other hand, the K409R alteration was used as alteration reported to be able to efficiently cause Fab arm exchange reaction. In this case, the amino acid residue at position 409 is Arg, probably leading to the stability problem under an acidic condition. Accordingly, this study was conducted to test whether or not to induce Fab arm exchange by introducing only association interface-controlling alteration reported in WO2006/106905 to a completely naturally occurring human IgG1 antibody without introducing K409R alteration.

The combinations of association interface-controlling alterations studied are shown in Table 1.

[0143]

[Table 1]

Antibody H chain gene name	SEQ ID NO	Alteration introduced to MRAH-G1d	Antibody H chain gene name	SEQ ID NO	Alteration introduced to H54-G1d
MRAH-G1dP1	17	D356K	H54-G1dN1	22	K439E
MRAH-G1dP3	18	D399K	H54-G1dN3	23	K409D
MRAH-G1dP4	19	E357K	H54-G1dN4	24	K370E
MRAH-G1dP5	20	D356K/D399K	H54-G1dN5	25	K409D/K439E
MRAH-G1dP6	21	D356K/E357K	H54-G1dN6	26	K370E/K439E

[0144]

Antibody L chains MRAL-k0 and L28-k0 were used for the H chain variable regions MRAH and H54, respectively. MRAH-G1dP1/MRAL-k0, H54-G1dN1/L28-k0, MRAH-G1dP3/MRAL-k0, H54-G1dN3/L28-k0, MRAH-G1dP4/MRAL-k0, H54-G1dN4/L28-k0, MRAH-G1dP5/MRAL-k0, H54-G1dN5/L28-k0, MRAH-G1dP6/MRAL-k0, and H54-G1dN6/L28-k0 were expressed and purified according to the method of Reference Example 1.

[0145]

Next, two types of homo variants thus obtained were mixed in the combinations given below, and the reaction products were evaluated according to the method of Reference Example 2.

(1) MRAH-G1dP1/MRAL-k0 and H54-G1dN1/L28-k0

(2) MRAH-G1dP3/MRAL-k0 and H54-G1dN3/L28-k0

(3) MRAH-G1dP4/MRAL-k0 and H54-G1dN4/L28-k0

(4) MRAH-G1dP5/MRAL-k0 and H54-G1dN5/L28-k0

(5) MRAH-G1dP6/MRAL-k0 and H54-G1dN6/L28-k0

Reaction condition: in TBS (Takara Bio Inc., pH 7.6), [each mAb] = 0.2 mg/ml, 0.05% Tween 20 (Junsei Chemical Co., Ltd.), [GSH (Sigma-Aldrich Corp.)] = 5 mM, 37°C, 24 hours.

The obtained results are shown in Table 2.

[0146]

[Table 2]

Abbreviation	Name of H chain constant region of MoAb1 used	Introduced alteration	Name of H chain constant region of MoAb2 used	Introduced alteration	Rate of bispecific antibody formation (%)
G1dP1/N1	G1dP1	D356K	G1dN1	K439E	1.7
G1dP3/N3	G1dP3	D399K	G1dN3	K409D	93.4
G1dP4/N4	G1dP4	E357K	G1dN4	K370E	4.4
G1dP5/N5	G1dP5	D356K/D399K	G1dN5	K409D/K439E	98.1
G1dP6/N6	G1dP6	D356K/E357K	G1dN6	K370E/K439E	29.3

[0147]

In the table, "Abbreviation" denotes the abbreviation of the homo variant combination used in the reaction. For example, the abbreviation G1dP1/N1 represents that MRAH-G1dP1/MRAL-k0 and H54-G1dN1/L28-k0 were reacted. "Name of H chain constant region of MoAb1 used" denotes the name of the constant region of the antibody having the variable region MRAH. "Name of H chain constant region of MoAb2 used" denotes the name of the constant region of the antibody having the variable region H54. "Introduced alteration" denotes the alteration introduced to MRAH-G1d or H54-G1d.

G1dP1/N1 with D356K introduced to one homo variant and K439E introduced to another homo variant had 1.7% rate of bispecific antibody formation. In Figure 2, G1drP1/N1 having K409R alteration and association interface-controlling alteration (D356K and K439E) had 99.3% rate of bispecific antibody formation under the same reaction conditions (5 mM GSH), showing that the reaction efficiency was drastically reduced in G1dP1/N1 instead containing Lys as the amino acid residue at EU numbering position 409. By contrast, G1dP3/N3 with association interface-controlling alteration D399K introduced to one homo variant and K409D introduced to another homo variant and G1dP5/N5 with D356K/D399K introduced to one homo variant and K409D/K439E introduced to another homo variant exhibited a rate of bispecific antibody formation as very high as 93.4% and 98.1%, respectively. These results demonstrated that Fab arm exchange can be induced with high efficiency by introducing only association interface-controlling alteration without the use of K409R alteration that gives an IgG4-type CH3 domain.

[0148]

Next, reaction efficiency was compared under 3 types of reducing conditions as to G1dP3/N3 and G1dP5/N5 having high reaction efficiency. In this comparison, G1drP1/N1 used in Example 2 and an altered form having the combination of K409R introduced to one antibody and F405L introduced to another homo variant as reported by Labrijn et al. as alterations for efficient bispecific antibody preparation by Fab arm exchange were also tested as controls (Labrijn AF et al., Proc. Natl., Acad. Sci., 2013. 110. 5145-5150).

K409R was introduced to MRAH-G1d to prepare MRAH-G1dr (SEQ ID NO: 27), and F405L was introduced to H54-G1d to prepare H54-G1dl (SEQ ID NO: 28). Antibody L chains MRAL-k0 and L28-k0 were used for the H chain variable regions MRAH and H54, respectively. MRAH-G1drP1/MRAL-k0, H54-G1drN1/L28-k0, MRAH-G1dP3/MRAL-k0, H54-G1dN3/L28-k0, MRAH-G1dP5/MRAL-k0, H54-G1dN5/L28-k0, MRAH-G1dr/MRAL-k0, and H54-G1dl/L28-k0 were expressed and purified according to the method of Reference Example 1.

[0149]

Next, two types of homo variants thus obtained were mixed in the combinations given below, and the reaction products were evaluated according to the method of Reference Example 2.

(1) MRAH-G1drP1/MRAL-k0 and H54-G1drN1/L28-k0

(2) MRAH-G1dP3/MRAL-k0 and H54-G1dN3/L28-k0

(3) MRAH-G1dP5/MRAL-k0 and H54-G1dN5/L28-k0

(4) MRAH-G1dr/MRAL-k0 and H54-G1dl/L28-k0

Reaction condition: in TBS (Takara Bio Inc., pH 7.6), [each mAb] = 0.2 mg/ml, 0.05% Tween 20 (Junsei Chemical Co., Ltd.), 37°C, 24 hours. The study was conducted under 3 conditions of a reducing agent [GSH (Sigma-Aldrich Corp.)] = 0.5 mM or 5 mM or [2-MEA (Sigma-Aldrich Corp.)] = 25 mM.

The obtained results are shown in Table 3.

[0150]

[Table 3]

Abbreviation	Name of H chain constant region of MoAb1 used	Introduced alteration	Name of H chain constant region of MoAb2 used	Introduced alteration	Rate of bispecific antibody formation (%)	Reductive condition
G1dr/l	G1dr	K409R	G1dl	F405L	87.3	GSH(5mM)
G1dP3/N3	G1dP3	D399K	G1dN3	K409D	85.2	GSH(5mM)
G1dP5/N5	G1dP5	D356K/D399K	G1dN5	K409D/K439E	99.1	GSH(5mM)
G1drP1/N1	G1drP1	D356K/K409R	G1drN1	K439E/K409R	99.3	GSH(5mM)
G1dr/l	G1dr	K409R	G1dl	F405L	95.6	2MEA(25mM)
G1dP3/N3	G1dP3	D399K	G1dN3	K409D	92.8	2MEA(25mM)
G1dP5/N5	G1dP5	D356K/D399K	G1dN5	K409D/K439E	100.0	2MEA(25mM)
G1drP1/N1	G1drP1	D356K/K409R	G1drN1	K439E/K409R	99.7	2MEA(25mM)
G1dr/l	G1dr	K409R	G1dl	F405L	9.5	GSH(0.5mM)
G1dR3/N3	G1dP3	D399K	G1dN3	K409D	4.8	GSH(0.5mM)
G1dP5/N5	G1dP5	D356K/D399K	G1dN5	K409D/K439E	75.4	GSH(0.5mM)
G1drP1/N1	G1drP1	D356K/K409R	G1drN1	K439E/K409R	21.8	GSH(0.5mM)

[0151]

In the table, "Abbreviation" denotes the abbreviation of the homo variant combination used in the reaction. For example, the abbreviation G1dP1/N1 represents that MRAH-G1dP1/MRAL-k0 and H54-G1dN1/L28-k0 were reacted. "Name of H chain constant region of MoAb1 used" denotes the name of the constant region of the antibody having the variable region MRAH. "Name of H chain constant region of MoAb2 used" denotes the name of the constant region of the antibody having the variable region H54. "Introduced alteration" denotes the alteration introduced to MRAH-G1d or H54-G1d.

[0152]

G1dr/l carrying the existing alteration to improve Fab arm exchange efficiency as reported by Labrijn et al. had 87.3% rate of bispecific antibody formation under the reducing condition of 5 mM GSH. Under this condition, G1dP3/N3 with D399K introduced to one homo variant and K409D introduced to another homo variant had 85.2% rate of bispecific antibody formation, and G1dP5/N5 with D356K/D399K introduced to one homo variant and K409D/K439E introduced to another homo variant had 99.1% rate of bispecific antibody formation. Also, G1drP1/N1 having D356K in one homo variant and K439E in another homo variant in addition to IgG4-type K409R alteration had 99.3% rate of bispecific antibody formation.

[0153]

G1dr/l carrying the existing alteration to improve Fab arm exchange efficiency as reported by Labrijn et al. had 95.6% rate of bispecific antibody formation under the reducing condition of 25 mM 2MEA. Under this condition, G1dP3/N3 with D399K introduced to one homo variant and K409D introduced to another homo variant had 92.8% rate of bispecific antibody formation, and G1dP5/N5 with D356K/D399K introduced to one homo variant and K409D/K439E introduced to another homo variant had 100% rate of bispecific antibody formation. Also, G1drP1/N1 having D356K in one homo variant and K439E in another homo variant in addition to IgG4-type K409R alteration had 99.7% rate of bispecific antibody formation.

[0154]

G1dr/l carrying the existing alteration to improve Fab arm exchange efficiency had 9.5% rate of bispecific antibody formation under the reducing condition of 0.5 mM GSH. Under this condition, G1dP3/N3 with D399K introduced to one homo variant and K409D introduced to another homo variant had 4.8% rate of bispecific antibody formation, and G1dP5/N5 with D356K/D399K introduced to one homo variant and K409D/K439E introduced to another homo variant had 75.4% rate of bispecific antibody formation. Also, G1drP1/N1 having D356K in one homo variant and K439E in another homo variant in addition to IgG4-type K409R alteration had 21.8% rate of bispecific antibody formation. This reducing condition drastically reduced

the rate of bispecific antibody formation in all of the samples as compared with the other reducing conditions.

[0155]

These results demonstrated that G1dP5/N5 with D356K/D399K introduced to one homo variant and K409D/K439E introduced to another homo variant exhibits a higher rate of bispecific antibody formation under all of the reaction conditions as compared with the existing alteration to improve Fab arm exchange efficiency as reported by Labrijn et al. The high rate of bispecific antibody formation is very important for the actual production of bispecific antibodies as drugs. Thus, this alteration is considered to be highly useful as compared with the existing alteration.

[0156]

[Example 4] Development of highly efficient Fab arm exchange using alteration for CH3 domain destabilization

The preceding Examples showed that provided that different charges are introduced to two types of homo variants by association interface-controlling alteration, a half-molecule formed from one homo variant in the presence of a reducing agent associates preferentially with a half-molecule derived from the other homo variant to form a bispecific antibody with high efficiency. Meanwhile, in the process of forming bispecific antibodies by Fab arm exchange, the dissociation of CH3 domains that forms half-molecules (HL molecules) after cleavage of two types of homo variants with a reducing agent reportedly becomes a rate-determining stage (Rispens T et al., J. Am. Chem. Soc., 2011. 133. 10302-10311). In short, if the dissociation of CH3 domains can be promoted by the moderate destabilization of the CH3 domains of each homo variant, Fab arm exchange can be expected to be induced more efficiently. Accordingly, the relationship between the rate of bispecific antibody formation and the stability of the CH3 domains of each homo variant was first evaluated in the presence of 5 mM GSH shown in Tables 2 and 3. The stability of the CH3 domains was determined with Tm (intermediate temperature of thermal denaturation) measured according to the method of Reference Example 3 as an index.

[0157]

Figure 3 shows the relationship between the rate of bispecific antibody formation and the value of higher Tm of CH3 in two types of homo variants used. G1dP1/N1 or G1dP4/N4 having a low rate of bispecific antibody formation had a CH3 Tm as high as 76°C or higher, whereas G1dP5/N5, G1dP3/N3, and G1drP1/N1 having high reaction efficiency had a homo variant CH3 Tm of 65.1°C, 69.6°C, and 69.5°C, respectively. These results revealed that the rate of bispecific antibody formation correlates evidently with the CH3 stability of each homo variant in Fab arm exchange. For achieving high reaction efficiency, it was also found preferable to destabilize the stability of the CH3 regions of a homo variant having more stable

CH3 between two types of homo variants used such that Tm of this CH3 falls below 70°C, etc. In this context, Tm of the CH3 regions (having the sequence of naturally occurring human IgG1) of MRAH-G1d/MRAL-k0 measured under the same condition as above was 83.6°C, demonstrating that for achieving high reaction efficiency in Fab arm exchange, it is required to destabilize the stability of the CH3 regions so as to decrease the Tm of the CH3 regions by, for example, 13°C or more from that of naturally occurring human IgG1.

[0158]

Thus, this study was conducted to test whether or not to improve the rate of bispecific antibody formation by decreasing the Tm of the CH3 domains of the homo variants used. IgG2-type alteration V397M was used as alteration to reduce the stability of CH3. IgG2 contains Met as an amino acid residue at EU numbering position 397. The introduction of the alteration to substitute this amino acid by IgG1-type Val reportedly improves the stability (Tm) of the CH3 regions (WO2009/041613). Thus, it was expected that the introduction of the V397M alteration to IgG1-type CH3 domains would destabilize the CH3 domains and facilitate their dissociation.

[0159]

Thus, V397M alteration was introduced to both homo variants of G1dP1/N1, G1dP4/N4, or G1dP6/N6 having a low rate of bispecific antibody formation in Table 2, and the resulting G1dP8/N8, G1dP9/N9, and G1dP10/N10 were studied. Specifically, V397M was introduced to MRAH-G1dP1, MRAH-G1dP4, MRAH-G1dP6, H54-G1dN1, H54-G1dN4, and H54-G1dN6 to prepare MRAH-G1dP8 (SEQ ID NO: 29), MRAH-G1dP9 (SEQ ID NO: 30), MRAH-G1dP10 (SEQ ID NO: 31), H54-G1dN8 (SEQ ID NO: 32), H54-G1dN9 (SEQ ID NO: 33), and H54-G1dN10 (SEQ ID NO: 34). Antibody L chains MRAL-k0 and L28-k0 were used for the H chain variable regions MRAH and H54. MRAH-G1dP8/MRAL-k0, H54-G1dN8/L28-k0, MRAH-G1dP9/MRAL-k0, H54-G1dN9/L28-k0, MRAH-G1dP10/MRAL-k0, and H54-G1dN10/L28-k0 were expressed and purified according to the method of Reference Example 1. The Tm of the obtained antibodies was measured according to the method of Reference Example 3.

[0160]

Next, two types of homo variants thus obtained were mixed in the combinations given below, and the reaction products were evaluated according to the method of Reference Example 2.

(1) MRAH-G1dP8/MRAL-k0 and H54-G1dN8/L28-k0

(2) MRAH-G1dP9/MRAL-k0 and H54-G1dN9/L28-k0

(3) MRAH-G1dP10/MRAL-k0 and H54-G1dN10/L28-k0

(4) MRAH-G1dr/MRAL-k0 and H54-G1dl/L28-k0

Reaction condition: in TBS (Takara Bio Inc., pH 7.6), [each mAb] = 0.2 mg/ml, 0.05% Tween 20 (Junsei Chemical Co., Ltd.), [GSH (Sigma-Aldrich Corp.)] = 5 mM, 37°C, 24 hours.

The obtained results are shown in Table 4.

[0161]

[Table 4]

Abbreviation	Name of H chain constant region of MoAb1 used	Introduced alteration	Name of H chain constant region of MoAb2 used	Introduced alteration	Rate of bispecific antibody formation (%)	Tm of MoAb1 CH3 (°C)	Tm of MoAb2 CH3 (°C)
G1dr/l	G1dr	K409R	G1dl	F405L	88.1	70.7	69.7
G1dP8/N8	G1dP8	D356K/V397M	G1dN8	V397M/K439E	73.2	70.1	70.5
G1dP9/N9	G1dP9	E357K/V397M	G1dN9	K370E/V397M	67.3	67	71
G1dP10/N10	G1dP10	D356K/E357K/V397M	G1dN10	K370E/V397M/K439E	96.9	63.8	70.2
G1dP1/N1 (extracted from Table 2)	G1dP1	D356K	G1dN1	K439E	1.7	76.7	74.7
G1dP4/N4 (extracted from Table 2)	G1dP4	E357K	G1dN4	K370E	4.4	68.5	76.5
G1dP6/N6 (extracted from Table 2)	G1dP6	D356K/E357K	G1dN6	K370E/K439E	29.3	66	70.1

[0162]

In the table, "Tm of MoAb1 CH3" denotes the Tm of CH3 of the homo variant having the variable region MRAH. "Tm of MoAb2 CH3" denotes the Tm of CH3 of the homo variant having the variable region H54.

In G1dP8/N8 with V397M alteration introduced to both homo variants of G1dP1/N1, the Tm of CH3 was decreased by 6.6°C to 70.1°C for MoAb1 and decreased by 4.2°C to 70.5°C for MoAb2, and the rate of bispecific antibody formation was improved from 1.7% to 73.2%. In G1dP9/N9 with V397M alteration introduced to both homo variants of G1dP4/N4, the Tm of CH3 was decreased by 1.5°C to 67°C for MoAb1 and decreased by 5.5°C to 71°C for MoAb2, and the rate of bispecific antibody formation was improved from 4.4% to 67.3%. In G1dP10/N10 with V397M alteration introduced to both homo variants of G1dP6/N6, the Tm of CH3 was decreased by 2.2°C to 63.8°C for MoAb1, albeit with no change in the Tm of MoAb2 CH3, and the rate of bispecific antibody formation was improved from 29.3% to 96.9%. These results demonstrated that the bispecific antibody formation efficiency in Fab arm exchange is improved by decreasing the Tm of homo variant CH3 through V397M alteration. In this test, G1dr/l carrying the existing alteration to improve Fab arm exchange efficiency had 88.1% rate of bispecific antibody formation. Thus, G1dP10/N10 was found to be superior thereto in the rate of bispecific antibody formation.

[0163]

Thus, this study was conducted to test whether or not to further improve the rate of bispecific antibody formation by introducing alteration expected to produce a larger CH3-destabilizing effect to the neighborhood of effective EU numbering position 397. Figure 4 shows the EU numbering position 397 of the CH3 domains and its neighborhood using the reported X-ray crystallographic structural data (PDB: 3DO3) on IgG1.

[0164]

First, D399 on the A chain is considered to interact electrostatically with K392 on the B chain. Therefore, it is possible that the substitution of K392 by Asp or Glu in addition to V397M alteration can cause the electrostatic repulsion between these chains to further destabilize the interaction between the chains. It is also expected that the substitution of K392 by an amino acid having a branched side chain can further destabilize the association between these chains through steric hindrance with M397. Furthermore, the possibility was also expected that the substitution of the amino acid residue at EU numbering position 397 by a more bulky amino acid could suppress CH3-CH3 association more than V397M alteration. From these viewpoints, 7 types of antibody H chain genes shown in Table 5 were newly prepared on the basis of MRAH-G1dP1 and H54-G1dN1.

[0165]

[Table 5]

Antibody H chain gene name	SEQ ID NO	Alteration introduced to MRAH-G1d	Antibody H chain gene name	SEQ ID NO	Alteration introduced to H54-G1d
MRAH-G1dP14	35	D356K/K392D/V397M	H54-G1dN14	42	K392D/V397M/K439E
MRAH-G1dP15	36	D356K/K392E/V397M	H54-G1dN15	43	K392E/V397M/K439E
MRAH-G1dP16	37	D356K/V397F	H54-G1dN16	44	V397F/K439E
MRAH-G1dP17	38	D356K/V397Y	H54-G1dN17	45	V397Y/K439E
MRAH-G1dP18	39	D356K/K392T/V397M	H54-G1dN18	46	K392T/V397M/K439E
MRAH-G1dP19	40	D356K/K392V/V397M	H54-G1dN19	47	K392V/V397M/K439E
MRAH-G1dP20	41	D356K/K392I/V397M	H54-G1dN20	48	K392I/V397M/K439E

[0166]

Antibody L chains MRAL-k0 and L28-k0 were used for the H chain variable regions MRAH and H54, respectively. MRAH-G1dP14/MRAL-k0, H54-G1dN14/L28-k0, MRAH-G1dP15/MRAL-k0, H54-G1dN15/L28-k0, MRAH-G1dP16/MRAL-k0, H54-G1dN16/L28-k0, MRAH-G1dP17/MRAL-k0, H54-G1dN17/L28-k0, MRAH-G1dP18/MRAL-k0, H54-G1dN18/L28-k0, MRAH-G1dP19/MRAL-k0, H54-G1dN19/L28-k0, MRAH-G1dP20/MRAL-k0, and H54-G1dN20/L28-k0 were expressed and purified according to the method of Reference Example 1. The Tm of the obtained antibodies was measured according to the method of Reference Example 3.

[0167]

Next, two types of homo variants thus obtained were mixed in the combinations given below, and the reaction products were evaluated according to the method of Reference Example 2.

(1) MRAH-G1dP14/MRAL-k0 and H54-G1dN14/L28-k0
(2) MRAH-G1dP15/MRAL-k0 and H54-G1dN15/L28-k0
(3) MRAH-G1dP16/MRAL-k0 and H54-G1dN16/L28-k0
(4) MRAH-G1dP17/MRAL-k0 and H54-G1dN17/L28-k0
(5) MRAH-G1dP18/MRAL-k0 and H54-G1dN18/L28-k0
(6) MRAH-G1dP19/MRAL-k0 and H54-G1dN19/L28-k0
(7) MRAH-G1dP20/MRAL-k0 and H54-G1dN20/L28-k0
(8) MRAH-G1dr/MRAL-k0 and H54-G1dl/L28-k0
(9) MRAH-G1dP1/MRAL-k0 and H54-G1dN1/L28-k0
(10) MRAH-G1dP8/MRAL-k0 and H54-G1dN8/L28-k0

Reaction condition: in TBS (Takara Bio Inc., pH 7.6), [each mAb] = 0.2 mg/ml, 0.05% Tween 20 (Junsei Chemical Co., Ltd.), [2-MEA (Sigma-Aldrich Corp.)] = 25 mM, 37°C, 24 hours.

The obtained results are shown in Table 6.

[0168]

[Table 6]

Abbreviation	Name of H chain constant region of MoAb1 used	Introduced alteration	Name of H chain constant region of MoAb2 used	Introduced alteration	Rate of bispecific antibody formation (%)	Tm of MoAb1 CH3 (°C)	Tm of MoAb2 CH3 (°C)
G1dr/l	G1dr	K409R	G1dl	F405L	91.7	70.7	69.7
G1dP1/N1	G1dP1	D356K	G1dN1	K439E	0.1	76.7	74.7
G1dP8/N8	G1dP8	D356K/V397M	G1dN8	V397M/K439E	73.2	70.1	70.5
G1dP14/N14	G1dP14	D356K/K392D/V397M	G1dN14	K392D/V397M/K439E	96.5	68.3	69.2
G1dP15/N15	G1dP15	D356K/K392E/V397M	G1dN15	K392E/V397M/K439E	96.9	69.3	70.2
G1dP16/N16	G1dP16	D356K/V397F	G1dN16	V397F/K439E	96.5	69.1	69.7
G1dP17/N17	G1dP17	D356K/V397Y	G1dN17	V397Y/K439E	98.0	69.2	69.8
G1dP18/N18	G1dP18	D356K/K392T/V397M	G1dN18	K392T/V397M/K439E	98.9	70.1	70.8
G1dP19/N19	G1dP19	D356K/K392V/V397M	G1dN19	K392V/V397M/K439E	70.8	70.7	71.1
G1dP20/N20	G1dP20	D356K/K392I/V397M	G1dN20	K392I/V397M/K439E	54.3	70.5	71.2

[0169]

G1dP8/N8 with V397M alteration introduced to both homo variants of G1dP1/N1 had 73.2% rate of bispecific antibody formation. By contrast, the rate of bispecific antibody formation was largely improved to 96.5% for G1dP14/N14 with K392D introduced to both chains thereof, 96.9% for G1dP15/N15 with K392E introduced, and 98.9% for G1dP18/N18 with K392T introduced. Also, the rate of bispecific antibody formation was improved to 96.5%

for G1dP16/N16 with V397F introduced instead of V397M to G1dP1/N1 and 98% for G1dP17/N17 with V397Y introduced, as compared with V397M. This is probably because, as seen from the fact that the CH3 domains of MoAb1 and MoAb2 in G1dP8/N8 containing V397M had a Tm of 70.1°C and 70.5°C, respectively, while the Tm of the CH3 domains of MoAb1 and MoAb2 was 69.1°C and 69.7°C for G1dP16/N16 and 69.2°C and 69.8°C for G1dP17/N17, these alterations compared with the V397M alteration weakened the interaction between the CH3 domains of each homo variant and facilitated their dissociation as intended. In this test, G1dr/l carrying the existing alteration to improve Fab arm exchange efficiency had 91.7% rate of bispecific antibody formation. Thus, G1dP14/N14, G1dP15/N15, G1dP16/N16, G1dP17/N17, and G1dP18/N18 were found to be superior thereto in the rate of bispecific antibody formation.

In consideration of applicability to drug production, G1dP16/N16 (D356K/V397F and V397F/K439E) and G1dP17/N17 (D356K/V397Y and V397Y/K439E) are very useful because of their higher rates of bispecific antibody formation and smaller amounts of heterogeneous components as compared with the existing alteration to improve Fab arm exchange efficiency.

Figure 5 shows the relationship between the CH3 stability of the altered forms studied in Tables 3 and 6 and the rate of bispecific antibody formation using 25 mM 2MEA as a reducing agent. As shown in Figure 5, the CH3 stability of each homo variant used correlates evidently with Fab arm exchange efficiency. The high rate of bispecific antibody formation is achieved by destabilizing the stability of the CH3 regions of a homo variant having more stable CH3 between two types of homo variants used such that Tm of this CH3 falls below 70°C, etc.

[0170]

[Example 5] Study on reaction time

The relationship between reaction time and reaction efficiency was studied using G1dP17/N17 found in Example 4.

Reaction condition: in TBS (Takara Bio Inc., pH 7.6), [each mAb] = 1.0 mg/ml, [2-MEA (Sigma-Aldrich Corp.)] = 25 mM, 37°C, total amount = 50 μl.

After 90 minutes, 3 hours, or 24 hours, 450 μl of a 25 mM MES buffer solution (pH 5.0) cooled to 4°C was added to the reaction solution, which was further stored at 4°C to terminate the reaction. Then, the reaction efficiency was evaluated according to the method of Reference Example 2 (Figure 6).

[0171]

As shown in Figure 6, the rate of bispecific antibody formation of G1dP17/N17 was 94.6% in 90 minutes, 95.2% in 3 hours, and 97.4% in 24 hours in the presence of 25 mM 2-MEA. Thus, the reaction time of 90 minutes offered the rate of approximately 95%. These results demonstrated that this altered form exhibits sufficiently higher reaction efficiency than the rate

of bispecific antibody formation (Table 6) of G1dr/l with K409R alteration introduced to one chain and F405L alteration introduced to another chain.

[0172]

[Example 6] Evaluation of binding of altered form exhibiting highly efficient Fab arm exchange to human FcgR and human FcRn

The altered form G1dP17/N17 that exhibited highly efficient FAE efficiency in Example 4 was evaluated for its binding to human FcgR and human FcRn. First, MRAH-G1d/MRAL-k0 having the sequence of naturally occurring IgG1 and the altered form MRAH-G1dP17/MRAL-k0//H54-G1dN17/L28-k0 after Fab arm exchange were tested for their binding to human FcRn according to the method of Reference Example 5. The results of analyzing binding to human FcRn are shown in Table 7.

[0173]

[Table 7]

Abbreviation	KD for human FcRn (M)
G1d	2.1E-06
G1dP17/N17	1.9E-06

[0174]

The results shown in Table 7 demonstrated that the altered form MRAH-G1dP17/MRAL-k0//H54-G1dN17/L28-k0 prepared by Fab arm exchange has human FcRn-binding activity equivalent to that of naturally occurring IgG1.

[0175]

Next, the binding activity against human FcgR was evaluated according to the method of Reference Example 4. In this context, MRAH-G1d/MRAL-k0 having the sequence of naturally occurring IgG1, the altered form MRAH-G1dP17/MRAL-k0//H54-G1dN17/L28-k0 after Fab arm exchange, two types of homo variants before Fab arm exchange reaction (MRAH-G1dP17/MRAL-k0 and H54-G1dN17/L28-k0), and two types of homo variants lacking the alteration V397Y to destabilize CH3 domains (MRAH-G1dP1/MRAL-k0 and H54-G1dN1/L28-k0) were evaluated together. In Table 8, KD fold hFcgRIa, KD fold hFcgRIIaR, KD fold hFcgRIIaH, KD fold hFcgRIIb, and KD fold hFcgRIIIaV are values that indicate the relative binding activity of each altered form when KD of G1d for each FcgR is defined as 1.

[0176]

[Table 8]

Abbreviation	Introduced alteration	KD for hFcgRIa (M)	KD for hFcgRIIaR (M)	KD for hFcgRIIaH (M)	KD for hFcgRIIb (M)	KD for hFcgRIIIaV (M)	KD fold hFcgRIa	KD fold hFcgRIIaR	KD fold hFcgRIIaH	KD fold hFcgRIIb	KD fold hFcgRIIIaV
G1d		1.6E-10	1.6E-06	8.7E-07	6.2E-06	5.1E-07	1	1	1	1	1
G1dP17	D356K+V397Y	2.6E-10	6.5E-07	4.1E-07	2.7E-06	2.5E-07	0.6	2.4	2.1	2.3	2.0
G1dN17	K439E+V397Y	1.3E-10	5.9E-07	3.9E-07	2.4E-06	2.0E-07	1.3	2.6	2.2	2.6	2.5
G1dP1	D356K	1.6E-10	1.7E-06	9.9E-07	7.0E-06	5.1E-07	1.0	0.9	0.9	0.9	1.0
G1dN1	K439E	2.5E-10	1.5E-06	9.6E-07	7.1E-06	4.5E-07	0.6	1.0	0.9	0.9	1.1
G1dP17/N17	D356K+V397Y/K439E+V397Y	1.4E-10	5.8E-07	3.7E-07	2.5E-06	2.1E-07	1.1	2.7	2.3	2.5	2.5

[0177]

The binding of the altered form G1dP17/N17 after Fab arm exchange compared with the naturally occurring antibody was enhanced by 1.1 times for hFcgRIa, 2.7 times for hFcgRIIaR, 2.3 times for hFcgRIIaH, 2.5 times for hFcgRIIb, and 2.5 times for hFcgRIIIaV. In this context, the homo variants G1dP1 and G1dN1 before the introduction of CH3 domain-destabilizing V397Y bound to each FcgR with activity equivalent to that of the naturally occurring antibody. Also, the homo variants derived therefrom by the introduction of V397Y (G1dP17 and G1dN17) exhibited enhanced binding to each FcgR. Therefore, the V397Y alteration was found to enhance the binding to hFcgR.

These results demonstrated that G1dP17/N17 that achieves high Fab arm exchange efficiency does not impair the binding to human FcRn and human FcgR as compared with the naturally occurring antibody.

[0178]

[Example 7] Study on Fab arm exchange in culture supernatant

For the production of bispecific antibodies by Fab arm exchange, it is assumed that two types of homo variants are separately cultured and purified, followed by the Fab arm exchange. If the reaction can occur in a culture supernatant, the homo variant purification step can be omitted. Therefore, this approach is highly advantageous. Accordingly, this study was conducted to test whether or not to cause Fab arm exchange with high efficiency by mixing two types of homo variants with a reducing agent in a culture supernatant.

[0179]

First, an amino acid residue at position 356 in MRAH-G1d and H54-G1d was substituted by E, and an amino acid residue at position 358 therein was substituted by M to prepare MRAH-G1m (SEQ ID NO: 49), H54-G1m (SEQ ID NO: 50), respectively. Next, E356K and K409R were introduced to MRAH-G1m to prepare MRAH-G1mrP1 (SEQ ID NO: 51). K439E and K409R were introduced to H54-G1m to prepare H54-G1mrN1 (SEQ ID NO: 52). Antibody L chains MRAL-k0 and L28-k0 were used for the H chain variable regions MRAH and H54, respectively. MRAH-G1mrP1/MRAL-k0 and H54-G1mrN1/L28-k0 were expressed and purified according to the method of Reference Example 1.

[0180]

FreeStyle 293 cells (Invitrogen Corp.) were cultured in FreeStyle 293 Expression medium and then centrifuged to recover a supernatant, which was then filtered through a 0.22 μm filtration membrane and used as Mock CM in Fab arm exchange.

Reaction condition: in Mock CM (pH 7.6), [each mAb] = 1.0 mg/ml, [2-MEA (Sigma-Aldrich Corp.)] = 25 mM, 37°C, 90 minutes

After the reaction, rProtein A Sepharose Fast Flow (GE Healthcare Japan Corp.) was added to the reaction solution for purification. Then, the reaction efficiency was evaluated according to the method of Reference Example 2 (Figure 7).

[0181]

As shown in Figure 7, the bispecific antibody was shown to be formed with 98% or higher reaction efficiency through the reaction at 37°C for 90 minutes in the presence of 25 mM 2-MEA even in the culture supernatant.

[0182]

[Example 8] Development of Fab arm exchange in mouse IgG1

The preceding Examples showed that Fab arm exchange is efficiently induced in human IgG1 or human IgG4. This study was conducted to test whether or not to similarly form a bispecific antibody by Fab arm exchange in mouse IgG1.

From the reported crystallographic structure (Harris LJ et al., J. Mol. Biol., 1998. 275. 861-872), D at EU numbering position 399 and K at EU numbering position 409 were presumed to contribute to the inter-chain interaction between CH3 domains (Figure 8). Accordingly, this study was conducted to test whether or not to induce Fab arm exchange by introducing charges for promoting heterodimerization to these sites, as in human IgG1.

[0183]

The antibody H chain variable regions used were H chain variable regions WT(H) (SEQ ID NO: 1; hereinafter, referred to as MRAH) and H54 (SEQ ID NO: 2) of the antibody against human interleukin 6 receptor disclosed in WO2009/125825. MRAH-mIgG1 (SEQ ID NO: 53) and H54-mIgG1 (SEQ ID NO: 54) having a mouse IgG1 H chain constant region as an antibody H chain constant region were prepared using the H chain variable regions. In addition, D399K was introduced as association interface-controlling alteration to MRAH-mIgG1 to prepare MRAH-mIgG1mP3 (SEQ ID NO: 55). D399R was introduced as association interface-controlling alteration to MRAH-mIgG1 to prepare MRAH-mIgG1mP4 (SEQ ID NO: 56). K409D was introduced as association interface-controlling alteration to H54-mIgG1 to prepare H54-mIgG1mN3 (SEQ ID NO: 57). K409E was introduced as association interface-controlling alteration to H54-mIgG1 to prepare H54-mIgG1mN4 (SEQ ID NO: 58). MRAL-mk1 (SEQ ID NO: 59) and L28-mk1 (SEQ ID NO: 60) having the sequence of a mouse κ chain constant region were prepared as L chains. The antibody L chains MRAL-mk1 and L28-mk1 were used for the H chain variable regions MRAH and H54, respectively. MRAH-mIgG1mP3/MRAL-mk1, MRAH-mIgG1mP4/MRAL-mk1, H54-mIgG1mN3/L28-mk1, and H54-mIgG1mN4/L28-mk1 were expressed and purified according to the method of Reference Example 1.

[0184]

Next, Fab arm exchange was carried out using the following combinations:

(1) MRAH-mIgG1mP3/MRAL-mk1 and H54-mIgG1mN3/L28-mk1
(2) MRAH-mIgG1mP4/MRAL-mk1 and H54-mIgG1mN4/L28-mk1

Reaction condition: in TBS (Takara Bio Inc., pH 7.6), [each mAb] = 2 mg/ml, [2-MEA (Sigma-Aldrich Corp.)] = 25 mM, 37°C, 19 hours.

After the reaction, the reaction efficiency was determined by CE-IEF according to the method of Reference Example 5 (Figure 9).

[0185]

As a result, the bispecific antibody was confirmed to be formed with efficiency as high as 89.2% by the reaction of MRAH-mIgG1mP3/MRAL-mk1 and H54-mIgG1mN3/L28-mk1 and 89.9% by the reaction of MRAH-mIgG1mP4/MRAL-mk1 and H54-mIgG1mN4/L28-mk1. This reaction efficiency was slightly lower than that of the Fab arm exchange using human IgG1 or human IgG4. This is presumably because mouse IgG1 has 3 disulfide bonds in the hinge regions, resulting in stronger binding between two heavy chains than that in the human IgG1 or human IgG4 hinges (Harris LJ et al., J. Mol. Biol., 1998. 275. 861-872).

[0186]

[Example 9] Evaluation of binding activity of bispecific antibody prepared by Fab arm exchange of mouse IgG1 against mouse FcgR and mouse FcRn

The two types of bispecific antibodies (MRAH-mIgG1mP3/MRAL-mk1//H54-mIgG1mN3/L28-mk1 and MRAH-mIgG1mP4/MRAL-mk1//H54-mIgG1mN4/L28-mk1) prepared by mouse IgG-type Fab arm exchange were tested for their binding to mouse FcgR and mouse FcRn according to Reference Example 4-3. Also, MRAH-mIgG1/MRAL-mk1 was prepared according to Reference Example 1 and assayed as a control. The assay results are shown in Table 9.

[0187]

[Table 9]

Abbreviation	KD for mFcgRI (M)	KD for mFcgRII (M)	KD for mFcgRIII (M)	KD for hFcgRIV (M)	KD fold mFcgRII	KD fold mFcgRIII
mIgG1	N.D.	8.2E-07	7.9E-07	N.D.	1.0	1.0
mIgG1mP3/mN3	N.D.	6.7E-07	6.7E-07	N.D.	1.2	1.2
mIgG1mP4/mN4	N.D.	6.7E-07	6.8E-07	N.D.	1.2	1.2

[0188]

Both of the two types of bispecific antibodies prepared exhibited a binding profile similar to that of naturally occurring mIgG1. Specifically, these bispecific antibodies exhibited 1.2 times the binding activity of naturally occurring mIgG1 against mFcgRII and mFcgRIII, without binding to mFcgRI and mFcgRIV.

[0189]

Next, the binding to mFcRn was evaluated according to Reference Example 4-4. The results are shown in Table 10.

[0190]

[Table 10]

Abbreviation	KD for mouse FcRn (M)	fold KD
mIgG1	2.5E-06	1.0
mP3/mN3	2.1E-06	1.2
mP4/mN4	1.9E-06	1.3

[0191]

Both of the two types of bispecific antibodies prepared were found to maintain mFcRn binding equivalent to that of naturally occurring mIgG1.

[0192]

[Example 10] Measurement of cytotoxic activity

Whether each human IgG-type bispecific antibody and mouse IgG-type bispecific antibody prepared by Fab arm exchange would maintain functions equivalent to those of a bispecific antibody prepared by an existing approach was evaluated by measuring the cytotoxic activity of an anti-human glypican 3 and anti-human CD3 bispecific antibody. First, an anti-human GPC3/anti-human CD3 bispecific antibody having human IgG4 constant regions was prepared as a control by the CrossMab technology reported by Schaefer et al. (Proc Natl Acad Sci, 2011, 108, 11187-11192). This molecule prepared by the CrossMab technology was a molecule in which the VH domain and the VL domain were exchanged within Fab against human GPC3 as described in WO2012/073985. The Knobs-into-Holes technology was used in an antibody H chain constant region in order to promote the hetero-association. The Knobs-into-Holes technology is a technique which involves substituting an amino acid side chain present in the CH3 region of one H chain with a larger side chain (knob), and substituting its counterpart amino acid side chain present in the CH3 region of another H chain with a smaller side chain (hole) so that the knob is inserted into the hole to promote the heterodimerization of the H chains, whereby the heterodimerized antibody of interest can be efficiently obtained (Nature, 1994, 372, 379-383). The alteration described in WO2011/108714 was used as alteration to attenuate binding to FcgR. Specifically, this alteration was introduced to substitute amino acid residues at EU numbering positions 234, 235, and 297 with Ala. Gly at EU numbering position 446 and Lys at EU numbering position 447 were removed from the C termini of the antibody H chains. In order to facilitate purification after antibody expression, a histidine tag was further added to the C terminus of the anti-human GPC3 H chain, and a FLAG

tag was further added to the C terminus of the anti-human CD3 H chain. GC33(2)H-G4dKnHS (SEQ ID NO: 61) was prepared as the anti-human GPC3 H chain thus altered. Also, rCE115H-G4dHlFS (SEQ ID NO: 62) was prepared as the anti-human CD3 H chain. Antibody L chains GC33(2)L-k0 (SEQ ID NO: 63) and rCE115L-k0 (SEQ ID NO: 64) were used on the anti-human GPC3 side and the anti-human CD3 side, respectively. The resulting antibody was expressed by transient expression in FreeStyle 293 cells according to Reference Example 1. The obtained culture supernatant was added to MabSelect SuRe column (GE Healthcare Japan Corp.), and the column was washed, followed by elution with 50 mM acetic acid. The fraction containing the antibody was added to HisTrap HP column (GE Healthcare Japan Corp.) or Ni Sepharose FF column (GE Healthcare Japan Corp.), and the column was washed, followed by elution with imidazole. The fraction containing the antibody was concentrated through an ultrafiltration membrane. Then, the concentrate was added to Superdex 200 column (GE Healthcare Japan Corp.). Only a monomeric antibody in the eluate was recovered to obtain a purified antibody GPC3 ERY22-rCE115.

[0193]

Next, each bispecific antibody having human IgG1-type, human IgG4-type, or mouse IgG1-type constant regions and anti-human GPC3/anti-human CD3 variable regions was prepared by Fab arm exchange. For the human IgG1-type and human IgG4-type H chain constant regions, the alteration to substitute an amino acid residue at EU numbering position 235 with Arg and an amino acid residue at EU numbering position 239 with Lys was introduced as FcgR binding-reducing alteration to G1dP17, G1dN17, G1drP1, G1drN1, G4dP1, and G4dN1 containing the alteration for Fab arm exchange to prepare F760P17, F760N17, F760G1drP1, F760G1drN1, F760G4dP1, and F760G4dN1, respectively. For the mouse IgG1-type H chain constant region, the alteration to substitute amino acid residues at EU numbering positions 235 and 239 with Lys was introduced as FcgR binding-reducing alteration to mIgG1mP4 and mIgG1mN4 used in Example 8 to prepare mF18mP4 and mF18mN4, respectively. The anti-human GPC3 sequence described in WO2012/073985 was used as a variable region to prepare H0000-F760N17 (SEQ ID NO: 65), H0000-F760G1drN1 (SEQ ID NO: 66), H0000-F760G4dN1 (SEQ ID NO: 67), and H0000-mF18mN4 (SEQ ID NO: 68). On the other hand, rCE115H-F760P17 (SEQ ID NO: 69), rCE115H-F760G1drP1 (SEQ ID NO: 70), rCE115H-F760G4dP1 (SEQ ID NO: 71), and rCE115H-mF18mP4 (SEQ ID NO: 72) were prepared as human CD3 side H chains. GL4-k0 (SEQ ID NO: 79) on the anti-human GPC3 side and rCE115L-k0 (SEQ ID NO: 64) on the anti-human CD3 side were commonly used as human IgG1-type and human IgG4-type antibody L chains. GL4-mk1 (SEQ ID NO: 80) on the anti-human GPC3 side and rCE115L-mk1 (SEQ ID NO: 81) on the anti-human CD3 side were used as mouse IgG1-type antibody L chains. These homo variants were expressed and purified according to the method

of Reference Example 1 to obtain rCE115H-F760P17/rCE115L-k0, H0000-F760N17/GL4-k0, rCE115H-F760G1drP1/rCE115L-k0, H0000-F760G1drN1/GL4-k0, rCE115H-F760G4dP1/rCE115L-k0, H0000-F760G4dN1/GL4-k0, rCE115H-mF18mP4/rCE115L-mk1, and H0000-mF18mP4/GL4-mk1.

[0194]

Next, two types of homo variants thus obtained were mixed in the combinations given below to cause FAE reaction.

(1) rCE115H-F760P17/rCE115L-k0 and H0000-F760N17/GL4-k0

(2) rCE115H-F760G1drP1/rCE115L-k0 and H0000-F760G1drN1/GL4-k0

(3) rCE115H-F760G4dP1/rCE115L-k0 and H0000-F760G4dN1/GL4-k0

(4) rCE115H-mF18mP4/rCE115L-mk1 and H0000-mF18mP4/GL4-mk1

Reaction condition: in TBS (Takara Bio Inc., pH 7.6), [each mAb] = 0.36 mg/ml, [2-MEA (Sigma-Aldrich Corp.)] = 25 mM, 37°C, 18 hours.

After the reaction, the products were dialyzed against PBS and used in the evaluation of cytotoxic activity.

The evaluation of cytotoxic activity was carried out by the method described in Reference Example 6. The results are shown in Figures 10-1 and 10-2.

[0195]

As shown in Figure 10-1, all of the bispecific antibodies prepared by human IgG1-type and human IgG4-type Fab arm exchange exhibited cytotoxic activity equivalent to that of the control antibody (GPC3 ERY22-rCE115) prepared by the existing bispecific antibody preparation technique. As shown in Figure 10-2, the bispecific antibody prepared by mouse IgG-type Fab arm exchange also exhibited cytotoxic activity equivalent to that of the control antibody (GPC3 ERY22-rCE115) prepared by the existing bispecific antibody preparation technique.

[0196]

[Example 11] Normal mouse PK test

(11-1) *In vivo* test using normal mouse

The *in vivo* test using a normal mouse was conducted to evaluate whether antibodies prepared by human IgG-type and mouse IgG-type Fab arm exchange would exhibit change in concentration in blood at the same level as in an antibody prepared by the existing approach.

Three types of anti-human glypican 3/anti-human CD3 bispecific antibodies TR-G1drP1/N1, TR-G1dP17/N17, and TR-G4dP1/N1 were prepared as human IgG-type antibodies by human IgG-type Fab arm exchange. Also, bispecific antibodies TR-G1dKiH and TR-G4dKiH having the same anti-human glypican 3/anti-human CD3 variable regions as above were prepared using constant regions prepared by the introduction of Knobs-into-Holes

alteration (Nature, 1994, 372, 379-383) to a constant region G1d starting at Ala at EU numbering position 118 in MRAH-G1d (SEQ ID NO: 3) or a constant region G4d (constant region wtG4d starting at Ala at EU numbering position 118 in MRAH-wtG4d (SEQ ID NO: 5) and further containing an IgG1-type hinge resulting from the substitution of an amino acid residue Ser at position 228 by Pro), and used as control antibodies. In this context, the constant region names G1dKiH and G4dKiH each denote constant regions expressed as a Knob chain and a Hole chain in combination using a Knob chain in which the knob alteration (alteration to substitute an amino acid residue at position 349 by Cys and an amino acid residue at position 366 by Trp) was introduced in the constant region G1d or G4d and a Hole chain in which the Hole alteration (alteration to substitute an amino acid residue at position 356 by Cys, an amino acid residue at position 366 by Ser, an amino acid residue at position 368 by Ala, and an amino acid residue at position 407 by Val) was introduced in the constant region G1d or G4d.

[0197]

On the other hand, H237-mIgG1mP3 (SEQ ID NO: 74), H237-mIgG1mN3 (SEQ ID NO: 75), H237-mIgG1mP4 (SEQ ID NO: 76), and H237-mIgG1mN4 (SEQ ID NO: 77) were prepared as mouse IgG-type antibodies by introducing alteration for Fab arm exchange to H237-mIgG1 (SEQ ID NO: 73) having the sequence of an H chain variable region H237 of the anti-human IL-6 receptor antibody described in WO2009/125825 and the sequence of a naturally occurring mIgG1 constant region. L104-mk1 (SEQ ID NO: 78) consisting of the sequence of an anti-human IL-6 receptor L chain variable region L104 and a mouse κ chain constant region mk1 was used as an antibody L chain. These homo variants were expressed according to the method of Reference Example 1 to obtain H237-mIgG1mP3/L104-mk1, H237-mIgG1mN3/L104-mk1, H237-mIgG1mP4/L104-mk1, and H237-mIgG1mN4/L104-mk1. Fab arm exchange was carried out using the obtained homo variants to obtain SA-mIgG1mP3/mN3 (H237-mIgG1mP3/L104-mk1 and H237-mIgG1mN3/L104-mk1 in combination) and SA-mIgG1mP4/mN4 (H237-mIgG1mP4/L104-mk1 and H237-mIgG1mN4/L104-mk1 in combination). SA-mIgG1 expressed using H237-mIgG1 and L104-mk1 was used as a control antibody.

[0198]

The Fab arm exchange was conducted under reaction conditions given below in all cases. After the reaction, the products were dialyzed against PBS and used in the *in vivo* test.

Reaction condition: in TBS (Takara Bio Inc., pH 7.6), [each mAb] = 0.225 mg/ml, [2-MEA (Sigma-Aldrich Corp.)] = 25 mM, 37°C, 17 hours.

[0199]

Each human IgG-type anti-human glypican 3/anti-human CD3 bispecific antibody (TR-G1dKiH, TR-G1drP1/N1, TR-G1dP17/N17, TR-G4dKiH, and TR-G4dP1/N1) or each anti-

human IL-6 receptor mouse antibody (SA-mIgG1, SA-mIgG1mP3/mN3, and SA-mIgG1mP4/mN4) was administered to a normal mouse (C57BL/6J mouse, Charles River Laboratories Japan, Inc.). Then, each antibody was evaluated for its *in vivo* kinetics. The antibody was adjusted to 0.1 mg/mL and administered at 10 mL/kg to the tail vein. After a lapse of 5 minutes, 2 hours, 1 day, 2 days, 3 days, 7 days, 14 days, 21 days, and 28 days after the antibody administration, blood was collected from the mouse. The collected blood was immediately centrifuged at 15,000 rpm at 4°C for 15 minutes to obtain plasma. The separated plasma was stored in a freezer set to -20°C or lower until the start of the assay.
[0200]

(11-2) Measurement of bispecific antibody concentration in plasma by ECLIA

The bispecific antibody concentration in the mouse plasma was measured by ECLIA. First, soluble human glypican 3 was dispensed to wells of MULTI-ARRAY 96-well Plate (Meso Scale Discovery) and left standing overnight at 4°C to prepare a soluble human glypican 3-immobilized plate. Calibration samples containing each bispecific antibody at 200, 100, 50, 25, 12.5, 6.25, or 3.125 ng/mL as a plasma concentration and mouse plasma assay samples diluted 100-fold or more were prepared. These calibration samples and plasma assay samples were dispensed at 100 μL/well to the soluble human glypican 3-immobilized plate and stirred at room temperature for 2 hours. Subsequently, a rabbit idiotype antibody against an anti-human CD3 antibody was stirred in the plate at room temperature for 1 hour. Then, Anti-Rabbit IgG-Sulfotag antibody (Meso Scale Discovery) was reacted therewith at room temperature for 1 hour. After addition of Read Buffer T (Meso Scale Discovery), light emission was measured using SECTOR Imager 2400 (Meso Scale Discovery). The antibody concentration in the mouse plasma was calculated from emission signals in the calibration curve using analysis software SOFTmax PRO (Molecular Devices). The results are shown in Figure 11. PK parameters are shown in Table 11. The results shown in Figure 11 and Table 11 demonstrated that all of the bispecific antibodies prepared by human IgG-type Fab arm exchange exhibit change in concentration in blood at the same level as in the control antibody prepared using the Knobs-into-Holes technology as the existing bispecific antibody preparation technique.
[0201]
[Table 11]

Abbreviation	t1/2 (days)	CL (mL/day/kg)	Vss (mL/kg)
TR-G1dKiH	17	3.49	84.5
TR-G1drP1/N1	15.3	3.98	83.5
TR-G1dP17/N17	16.9	3.05	71.5
TR-G4dKiH	19.5	3.05	84.9
TR-G4dP1/N1	23.7	2.22	73.5

[0202]

(11-3) Measurement of anti-human IL-6 receptor mouse antibody concentration in plasma by ELISA

The anti-human IL-6 receptor mouse antibody concentration in the mouse plasma was measured by ELISA. First, soluble human IL-6 receptor was dispensed to wells of Nunc-Immuno Plate, MaxiSoup (Nalge Nunc International Corp.) and left standing overnight at 4°C to prepare a soluble human IL-6 receptor-immobilized plate. Calibration samples containing each anti-human IL-6 receptor mouse antibody at 2.50, 1.25, 0.625, 0.313, 0.156, 0.078, or 0.039 μg/mL as a plasma concentration and mouse plasma assay samples diluted 100-fold or more were prepared. These calibration samples and plasma assay samples were dispensed at 100 μL/well to the soluble human IL-6 receptor-immobilized plate and stirred at room temperature for 2 hours. Then, Anti-Mouse IgG-Peroxidase antibody (Sigma-Aldrich Corp.) was reacted therewith at room temperature for 2 hours, and the color reaction of the reaction solution was carried out using TMB One Component HRP Microwell Substrate (BioFX Laboratories, Inc.) as a substrate. The reaction was terminated by the addition of 1 N sulfuric acid (Showa Chemical Industry Co., Ltd.). The absorbance of the reaction solution in each well was measured at 450 nm using a microplate reader. The antibody concentration in the mouse plasma was calculated from the absorbance in the calibration curve using analysis software SOFTmax PRO (Molecular Devices). The results are shown in Figure 12. The antibody parameters are shown in Table 12. The results shown in Figure 12 and Table 12 demonstrated that the antibodies prepared by mouse IgG-type Fab arm exchange exhibit change in concentration in blood at the same level as in the control antibody having the sequence of naturally occurring mIgG1.

[0203]

[Table 12]

Abbreviation	t1/2 (days)	CL (mL/day/kg)	Vss (mL/kg)
SA-mIgG1	12.8	5.13	98.7
SA- mIgG1mP3/mN3	16.6	3.7	86.7
SA- mIgG1mP4/mN4	21.9	3.47	104

[0204]

[Reference Example 1] Preparation of antibody expression vector and expression and purification of antibody

The full-length genes having nucleotide sequences encoding the H chain and the L chain of each antibody were synthesized using assembly PCR or the like and prepared by a method generally known to those skilled in the art. Amino acid substitution was introduced by a method generally known to those skilled in the art using PCR or the like. The obtained plasmid fragments were inserted to expression vectors for animal cells to prepare H chain expression vectors and L chain expression vectors. The nucleotide sequences of the obtained expression vectors were determined by a method generally known to those skilled in the art. The prepared plasmids were transiently transferred to a human embryonic kidney cancer cell-derived HEK293H line (Invitrogen Corp.) or FreeStyle 293 cells (Invitrogen Corp.) for antibody expression. The obtained culture supernatant was recovered and then passed through a 0.22 μm filter MILLEX(R)-GV (Millipore Corp.) or a 0.45 μm filter MILLEX(R)-GV (Millipore Corp.) to obtain a culture supernatant. The antibody was purified from the obtained culture supernatant by a method generally known to those skilled in the art using rProtein A Sepharose Fast Flow (GE Healthcare Japan Corp.) or Protein G Sepharose 4 Fast Flow (GE Healthcare Japan Corp.). As for the concentration of the purified antibody, the absorbance was measured at 280 nm using a spectrophotometer, and the antibody concentration was calculated by use of an extinction coefficient calculated from the obtained value by a method such as PACE (Protein Science 1995; 4: 2411-2423).

[0205]

[Reference Example 2] Evaluation of rate of bispecific antibody formation by ion-exchange chromatography

The separation of each specimen was evaluated by the ion-exchange chromatography purification method using Prominence UFLC (Shimadzu Corp.). The bispecific antibody was separated by the two-solution mixed gradient method using a 25 mM MES buffer solution (pH 5.0) and a 25 mM MES buffer solution (pH 5.0) containing 500 mM sodium chloride as mobile phases and ProPac WCX-10 (Thermo Fisher Scientific K.K.) as a column. The data was obtained at a wavelength of 215 nm. The elution results were evaluated using Empower 2 (Waters Corp.).

A value determined by dividing the area value of the bispecific antibody by the area value of all antibodies present in the system, followed by multiplication by 100 was used as the rate of bispecific antibody formation (%). If one of the homo variants had a poor rate of recovery, the area value of the other homo variant was doubled and summed with the area value of the

bispecific antibody, and the resulting value was used as the area value of all antibodies for the calculation.

[0206]

[Reference Example 3] Measurement of Tm

The Tm of CH3 domains was measured by a method generally known to those skilled in the art using Rotor-gene Q (Qiagen N.V.). A sample containing a mixture of each antibody at a concentration of 0.1 mg/mL and SYPRO orange at a concentration of 10 X concentrate was heated from 30°C to 99°C. The fluorescence intensity (excitation wavelength: 470 nm, fluorescence wavelength: 555 nm) was measured on the basis of 0.4°C. This measurement was conducted in PBS (Sigma-Aldrich Corp., pH 7.4). The analysis was conducted using Rotor-gene Q series software. The point of inflection determined by the first derivation of the fluorescence intensity was defined as Tm. The Tm of the CH3 domains was calculated through the use of Tm of MRAH CH2 around 70°C, Tm of MRAH Fab around 95°C, Tm of H54 CH2 around 70°C, and Tm of H54 Fab around 90°C.

[0207]

[Reference Example 4] Analysis of interaction by SPR

(4-1) Method for preparing FcγR and method for analyzing interaction between altered antibody and FcγR

The extracellular domain of each FcγR was prepared by the following method: first, the gene of the FcγR extracellular domain was synthesized by a method generally known to those skilled in the art. For this synthesis, the sequence of each FcγR was prepared on the basis of the information registered in NCBI. Specifically, FcγRI was prepared on the basis of the sequence of NCBI accession No. NM_000566.3; FcγRIIa was prepared on the basis of the sequence of NCBI accession No. NM_001136219.1; FcγRIIb was prepared on the basis of the sequence of NCBI accession No. NM_004001.3; FcγRIIIa was prepared on the basis of the sequence of NCBI accession No. NM_001127593.1; and FcγRIIIb was prepared on the basis of the sequence of NCBI accession No. NM_000570.3. These sequences were C-terminally tagged with a His tag sequence. Also, polymorphism is known about FcγRIIa, FcγRIIIa, and FcγRIIIb. The polymorphic sites were prepared with reference to J. Exp. Med., 1990, 172: 19-25 for FcγRIIa, J. Clin. Invest., 1997, 100 (5): 1059-1070 for FcγRIIIa, and J. Clin. Invest., 1989, 84, 1688-1691 for FcγRIIIb.

[0208]

Each obtained gene fragment was inserted to expression vectors for animal cells to prepare expression vectors. The prepared expression vectors were transiently transferred to human embryonic kidney cancer cell-derived FreeStyle 293 cells (Invitrogen Corp.) to express the protein of interest. After culture, the obtained culture supernatant was recovered and then

passed through a 0.22 μm filter to obtain a culture supernatant. The obtained culture supernatant was purified, as a rule, by the following 4 steps: cation-exchange column chromatography (SP Sepharose FF) as step 1, affinity column chromatography for the His tag (HisTrap HP) as step 2, gel filtration column chromatography (Superdex 200) as step 3, and sterile filtration as step 4. However, for FcγRI, anion-exchange column chromatography was carried out in step 1 using Q Sepharose FF. The absorbance was measured for each purified protein at 280 nm using a spectrophotometer, and the concentration of the purified protein was calculated by use of an extinction coefficient calculated from the obtained value by a method such as PACE (Protein Science 1995; 4: 2411-2423).

[0209]

Each altered antibody was analyzed for its interaction with each Fcγ receptor thus prepared using Biacore T100 (GE Healthcare Japan Corp.), Biacore T200 (GE Healthcare Japan Corp.), Biacore A100, or Biacore 4000. The running buffer used was HBS-EP+ (GE Healthcare Japan Corp.). The assay temperature was set to 25°C. The sensor chips used were chips prepared by immobilizing the antigenic peptide, Protein A (Thermo Fisher Scientific K.K.), Protein A/G (Thermo Fisher Scientific K.K.), or Protein L (ACTIGEN or BioVision) onto Series S Sensor Chip CM5 (GE Healthcare Japan Corp.) or Series S Sensor Chip CM4 (GE Healthcare Japan Corp.) by the amine coupling method, or by immobilizing the antigenic peptide biotinylated in advance onto Series S Sensor Chip SA (certified) (GE Healthcare Japan Corp.) through interaction.

[0210]

The antibody of interest was captured onto these sensor chips and allowed to interact with the Fcγ receptor diluted with a running buffer. The binding amount to the antibody was measured and compared among antibodies. Since the binding amount of the Fcγ receptor depends on the amount of the captured antibody, a correction value determined by dividing the binding amount of the Fcγ receptor by the amount of each captured antibody was used in the comparison. The antibody captured on the sensor chip was washed off through the reaction of 10 mM glycine-HCl (pH 1.5) to regenerate the sensor chip, which was repetitively used.

[0211]

In order to calculate the KD value of each altered antibody for FcγR, kinetic analysis was conducted according to the following method: first, the antibody of interest was captured onto these chips and allowed to interact with the Fcγ receptor diluted with a running buffer. For the obtained sensorgram, the assay results were globally fit into the 1:1 Langmuir binding model using Biacore Evaluation Software to calculate an association rate constant ka (L/mol/s) and a dissociation rate constant kd (1/s). From these values, the dissociation constant KD (mol/L) was calculated.

[0212]

(4-2) Method for preparing FcRn and method for analyzing interaction between altered antibody and FcRn

FcRn is a complex of FcRn and β2-microglobulin. Oligo DNA primers were prepared on the basis of the published gene sequence of human FcRn (J Exp Med. 1994 Dec 1; 180 (6): 2377-81). The DNA fragment containing the whole gene encoding the FcRn was prepared by PCR using the prepared primers and human cDNA (Human Placenta Marathon-Ready cDNA, Clontech Laboratories, Inc.) as a template. A DNA fragment encoding the extracellular domain containing a signal region (Met1 to Leu290) was amplified by PCR using the obtained DNA fragment as a template and inserted to expression vectors for mammalian cells. Likewise, oligo DNA primers were prepared on the basis of the published gene sequence of human β2-microglobulin (Proc. Natl. Acad. Sci. U.S.A. 99 (26): 16899-16903 (2002)). The DNA fragment containing the whole gene encoding the β2-microglobulin was prepared by PCR using the prepared primers and human cDNA (Human Placenta Marathon-Ready cDNA, Clontech Laboratories, Inc.) as a template. A DNA fragment encoding the whole protein containing a signal region (Met1 to Met119) was amplified by PCR using the obtained DNA fragment as a template and inserted to expression vectors for mammalian cells.

[0213]

Soluble human FcRn was expressed by the following procedures: the plasmids constructed for expressing human FcRn (SEQ ID NO: 30) and β2-microglobulin (SEQ ID NO: 31) were transferred to cells of a human embryonic kidney cancer cell-derived cell line HEK293H (Invitrogen Corp.) by lipofection using PEI (Polysciences, Inc.). The obtained culture supernatant was recovered and purified using IgG Sepharose 6 Fast Flow (Amersham Biosciences Corp.). Then, FcRn was further purified using HiTrap Q HP (GE Healthcare Japan Corp.) (J Immunol. 2002 Nov 1; 169 (9): 5171-80).

A system using an antibody immobilized on the sensor chip described in J Immunol. 2009; 182 (12): 7663-71 and human FcRn as an analyte has been reported as an assay system for evaluating the interaction between the antibody and FcRn using Biacore. For this purpose, human FcRn was prepared as described in Reference Example 4. This system was used to evaluate the binding activity (dissociation constant KD) of Fv4-IgG1, Fv4-IgG1-v1, and Fv4-IgG1-v2 against human FcRn at pH 6.0 and pH 7.4. Each antibody as a test substance was directly immobilized onto Series S Sensor Chip CM5 and subjected to the test. The immobilization of the antibody to the sensor chip was carried out using 50 mmol/L sodium phosphate, 150 mmol/L NaCl, and 0.05% (v/v%) Surfactant P20 (pH 6.0) as a running buffer and an amine coupling kit according to the manual of the manufacturer in order to attain 500 RU as the target amount of the antibody immobilized.

[0214]

The assay was conducted by use of the prepared sensor chip using 50 mmol/L sodium phosphate / 150 mmol/L NaCl, and 0.05% Surfactant P20 (pH 6.0) or 50 mmol/L sodium phosphate, 150 mmol/L NaCl, and 0.05% Surfactant P20 (pH 7.4) as a running buffer. The assay was conducted at 25°C for all samples. The diluted solution of the human FcRn or a running buffer (as a control solution) was injected thereto at a flow rate of 5 μL/min for 10 minutes so that the human FcRn was allowed to interact with the antibody on the sensor chip. Then, a running buffer was injected thereto at a flow rate of 5 μL/min for 1 minute. After observation of the dissociation of FcRn, 20 mmol/L Tris-HCl / 150 mmol/L NaCl (pH 8.1) were injected at a flow rate of 30 μL/min for 15 seconds, and this operation was repeated twice to regenerate the sensor chip.

[0215]

In order to calculate the KD value of each altered antibody for FcRn, kinetic analysis was conducted according to the following method: first, the antibody of interest was captured onto these chips and allowed to interact with FcRn diluted with a running buffer. For the obtained sensorgram, the assay results were globally fit into the 1:1 Langmuir binding model using Biacore Evaluation Software to calculate an association rate constant ka (L/mol/s) and a dissociation rate constant kd (1/s). From these values, the dissociation constant KD (mol/L) was calculated.

[0216]

(4-3) Method for preparing mFcγR and method for analyzing interaction between altered antibody and mFcγR

The extracellular domain of each mouse FcγR was prepared by the following method: first, the gene of the FcγR extracellular domain was synthesized by a method generally known to those skilled in the art. For this synthesis, the sequence of each FcγR was prepared on the basis of the information registered in NCBI. Specifically, mFcγRI was prepared on the basis of the sequence of NCBI Reference Sequence: NP_034316.1; mFcγRII was prepared on the basis of the sequence of NCBI Reference Sequence: NP_034317.1; mFcγRIII was prepared on the basis of the sequence of NCBI Reference Sequence: NP_034318.2; and mFcγRIV was prepared on the basis of the sequence of NCBI Reference Sequence: NP_653142.2. These sequences were C-terminally tagged with a His tag sequence.

[0217]

Each obtained gene fragment was inserted to expression vectors for animal cells to prepare expression vectors. The prepared expression vectors were transiently transferred to human embryonic kidney cancer cell-derived FreeStyle 293 cells (Invitrogen Corp.) to express the protein of interest. The obtained culture supernatant was recovered and then passed through

a 0.22 μm filter to obtain a culture supernatant. The obtained culture supernatant was purified, as a rule, by the following 4 steps: ion-exchange column chromatography as step 1, affinity column chromatography for the His tag (HisTrap HP) as step 2, gel filtration column chromatography (Superdex 200) as step 3, and sterile filtration as step 4. The ion-exchange column chromatography of step 1 was carried out using Q Sepharose HP for mFcγRI, SP Sepharose FF for mFcγRII and mFcγRIV, and SP Sepharose HP for mFcγRIII. D-PBS(-) was used as a solvent in step 3 or later, while D-PBS(-) containing 0.1 M arginine was used for mFcγRIII. The absorbance was measured for each purified protein at 280 nm using a spectrophotometer, and the concentration of the purified protein was calculated by use of an extinction coefficient calculated from the obtained value by a method such as PACE (Protein Science 1995; 4: 2411-2423).

[0218]

Each altered antibody was analyzed for its interaction with each Fcγ receptor thus prepared using Biacore T100 (GE Healthcare Japan Corp.), Biacore T200 (GE Healthcare Japan Corp.), Biacore A100, or Biacore 4000. The running buffer used was HBS-EP+ (GE Healthcare Japan Corp.). The assay temperature was set to 25°C. The sensor chips used were chips prepared by immobilizing the antigenic peptide, Protein A (Thermo Fisher Scientific K.K.), Protein A/G (Thermo Fisher Scientific K.K.), or Protein L (ACTIGEN or BioVision) onto Series S Sensor Chip CM5 (GE Healthcare Japan Corp.) or Series S Sensor Chip CM4 (GE Healthcare Japan Corp.) by the amine coupling method, or by immobilizing the antigenic peptide biotinylated in advance onto Series S Sensor Chip SA (certified) (GE Healthcare Japan Corp.) through interaction.

[0219]

The antibody of interest was captured onto these sensor chips and allowed to interact with mFcγR diluted with a running buffer. The binding amount to the antibody was measured and compared among antibodies. Since the binding amount of mFcγR depends on the amount of the captured antibody, a correction value determined by dividing the binding amount of mFcγR by the amount of each captured antibody was used in the comparison. The antibody captured on the sensor chip was washed off through the reaction of 10 mM glycine-HCl (pH 1.5) to regenerate the sensor chip, which was repetitively used.

[0220]

In order to calculate the KD value of each altered antibody for FcγR, kinetic analysis was conducted according to the following method: first, the antibody of interest was captured onto these chips and allowed to interact with mFcγR diluted with a running buffer. For the obtained sensorgram, the assay results were globally fit into the 1:1 Langmuir binding model using Biacore Evaluation Software to calculate an association rate constant ka (L/mol/s) and a

dissociation rate constant kd (1/s). From these values, the dissociation constant KD (mol/L) was calculated.

[0221]

(4-4) Method for preparing mFcRn and method for analyzing interaction between altered antibody and mFcRn

Kinetic analysis was conducted on mouse FcRn and each antibody using Biacore T100, Biacore T200, Biacore A100, and Biacore 4000 (GE Healthcare Japan Corp.). An appropriate amount of protein L (ACTIGEN) was immobilized on Sensor Chip CM4 (GE Healthcare Japan Corp.) by the amine coupling method. The antibody of interest was captured onto the chip. Next, a diluted FcRn solution or a running buffer (as a control solution) was injected thereto so that the mouse FcRn was allowed to interact with the antibody captured on the sensor chip. The running buffer used was 50 mmol/L sodium phosphate, 150 mmol/L NaCl, and 0.05% (w/v) Tween 20 (pH 6.0), and each buffer was also used for diluting FcRn. 10 mmol/L glycine-HCl (pH 1.5) was used to regenerate the chip. The assay was conducted at 25°C for all samples. From the sensorgram obtained by the assay, an association rate constant ka (1/Ms) and a dissociation rate constant k_d (1/s) were calculated as kinetic parameters. The KD (M) of each antibody for mouse FcRn was calculated on the basis of the parameters. Biacore Evaluation Software (GE Healthcare Japan Corp.) was used in the calculation of each parameter.

[0222]

[Reference Example 5] CE-IEF

The CE-IEF measurement was carried out by a method generally known to those skilled in the art using PA800 Plus (Beckman Coulter Inc.). Pharmalyte having a broad range of 5 to 8 and Pharmalyte having a broad range of 8 to 10.5 were mixed in equal amounts and analyzed in a pI range of 5 to 10.5. The analysis was conducted using a 4 mg/mL antibody solution, and the results were analyzed using 32 karat software (Beckman Coulter Inc.). A value determined by dividing the area value of the bispecific antibody by the area value of all antibodies present in the system, followed by multiplication by 100 was used as the rate of bispecific antibody formation (%).

[0223]

[Reference Example 6] Measurement of cytotoxic activity

(6-1) Preparation of human peripheral blood mononuclear cell (PBMC) solution

50 mL of peripheral blood was collected from each healthy volunteer (adult) using a syringe pre-filled with 100 μL of 1,000 units/mL of a heparin solution (Novo-Heparin 5,000 units for Injection, Novo Nordisk A/S). The peripheral blood was diluted 2-fold with PBS(-) and then divided into four equal parts, which were then added to Leucosep lymphocyte separation tubes (Cat. No. 227290, Greiner Bio-One GmbH) pre-filled with 15 mL of Ficoll-

Paque PLUS and centrifuged in advance. After centrifugation (2,150 rpm, 10 minutes, room temperature) of the separation tubes, a mononuclear cell fraction layer was separated. The cells in the mononuclear cell fraction were washed once with Dulbecco's Modified Eagle's Medium containing 10% FBS (Sigma-Aldrich Corp.; hereinafter, referred to as 10% FBS/D-MEM). Then, the cells were adjusted to a cell density of 4×10^6 cells/mL with 10% FBS/D-MEM. The cell solution thus prepared was used as a human PBMC solution in the subsequent test.

[0224]

(6-2) Measurement of cytotoxic activity

The cytotoxic activity was evaluated on the basis of the rate of cell growth inhibition using xCELLigence real-time cell analyzer (Roche Diagnostics). The target cells used were an SK-pca13a cell line established by forcing an SK-HEP-1 cell line to express human GPC3. SK-pca13a was dissociated from the dish and inoculated at 100 μL/well (1×10^4 cells/well) to an E-Plate 96 (Roche Diagnostics) plate to start the assay of live cells using the xCELLigence real-time cell analyzer. On the next day, the plate was taken out of the xCELLigence real-time cell analyzer, and 50 μL of each antibody adjusted to each concentration (0.004, 0.04, 0.4, and 4 μg/ml) was added to the plate. After reaction at room temperature for 15 minutes, 50 μL (2×10^4 cells/well) of the human PBMC solution prepared in the preceding paragraph (6-1) was added thereto. This plate was reloaded to the xCELLigence real-time cell analyzer to start the assay of live cells. The reaction was carried out under conditions of 5% CO_2 and 37°C. 72 hours after the addition of human PBMC. The rate of cell growth inhibition (%) was determined from the cell index value according to the expression given below. A numeric value after normalization against the cell index value immediately before the addition of the antibody defined as 1 was used as the cell index value in this calculation.

Rate of cell growth inhibition (%) = $(A - B) \times 100 / (A - 1)$, wherein

A represents the average cell index value of wells non-supplemented with the antibody (only the target cells and human PBMC), and B represents the average cell index value of the wells supplemented with each antibody.

Industrial Applicability

[0225]

Bispecific antibodies can be prepared under a reducing condition with higher efficiency by use of the method of the present invention than that by the conventional techniques.

Claims

1. A method for producing a heteromultimer, comprising the steps of:
a) providing a homo variant of first polypeptides each having a first antigen-binding activity and comprising an Fc region;
b) providing a homo variant of second polypeptides each having a second antigen-binding activity different from the first antigen-binding activity and comprising an Fc region;
c) incubating the homo variant of the first polypeptides and the homo variant of the second polypeptides together under a reducing condition that allows cysteines in hinge regions to cause disulfide bond isomerization; and
d) obtaining a heteromultimer comprising the first and second polypeptides, wherein

1 to 3 sets of amino acid residues selected from the following amino acid residue sets:

(1) amino acid residues at EU numbering positions 356 and 439,

(2) amino acid residues at EU numbering positions 357 and 370, and

(3) amino acid residues at EU numbering positions 399 and 409

in a CH3 region contained in the Fc region of the first and/or second polypeptide have the same type of charge,
wherein in the first and/or second polypeptide, an amino acid at EU numbering position 397 and/or 392 is substituted, and

when the amino acid residues in the same set among the amino acid residue sets (1) to (3) have the same type of charge as each other both in the CH3 region of the first polypeptide and in the CH3 region of the second polypeptide, the amino acid residues in this set in the CH3 region of the second polypeptide have a charge opposite to that of the amino acid residues in this set in the CH3 region of the first polypeptide.

2. The method according to claim 1, wherein the step a) in claim 1 comprises the step of providing a third polypeptide that forms a multimer with the first polypeptide, and the step b) comprises the step of providing a fourth polypeptide that forms a multimer with the second polypeptide.

3. The method according to claim 1 or 2, wherein the amino acid residues having the same type of charge are selected from one or more amino acid residues included in any of the following groups (A) and (B):
(A) glutamic acid (E) and aspartic acid (D); and
(B) lysine (K), arginine (R), and histidine (H).

4. The method according to any one of claims 1 to 3, wherein the set(s) of the amino acid residues having the same type of charge as each other in each of the first and second polypeptides is any one of the following amino acid residue sets (1) to (4):

(1) amino acid residues at EU numbering positions 356 and 439,

(2) amino acid residues at EU numbering positions 357 and 370,

(3) amino acid residues at EU numbering positions 399 and 409, and

(4) (i) amino acid residues at EU numbering positions 399 and 409 and

(ii) amino acid residues at EU numbering positions 356 and 439.

5. The method according to any one of claims 1 to 4, wherein the set(s) of the amino acid residues having the same type of charge as each other in each of the first and second polypeptides is the following amino acid residue sets:

(i) amino acid residues at EU numbering positions 399 and 409 and

(ii) amino acid residues at EU numbering positions 356 and 439.

6. The method according to any one of claims 1 to 5, wherein in the first and/or second polypeptide, an amino acid is altered so as to destabilize the stability of the CH3 region of the first and/or second polypeptide.

7. The method according to any one of claims 1 to 6, wherein the Fc region of the first and/or second polypeptide is of IgG1, IgG2, IgG3, or IgG4 type.

8. The method according to any one of claims 1 to 6, wherein the Fc region of the first and/or second polypeptide is a mouse-derived Fc region.

9. The method for producing a heteromultimer according to claim 8, wherein
1 to 3 sets of amino acid residues selected from the following amino acid residue sets:

(1) amino acid residues at EU numbering positions 356 and 439,

and

(2) amino acid residues at EU numbering positions 399 and 409

in the CH3 region contained in the Fc region of the first and/or second polypeptide have the same type of charge, and

when the amino acid residues in the same set among the amino acid residue sets (1) to (3) have the same type of charge as each other both in the CH3 region of the first polypeptide and in the CH3 region of the second polypeptide, the amino acid residues in this set in the CH3 region

of the second polypeptide have a charge opposite to that of the amino acid residues in this set in the CH3 region of the first polypeptide.

10. The method according to any one of claims 1 to 9, wherein in the first and/or second polypeptide,

the amino acid at EU numbering position 397 is altered to Met (M), Phe (F), or Tyr (Y), and/or

the amino acid at EU numbering position 392 is altered to Asp (D), Glu (E), Thr (T), Val (V), or Ile (I).

11. The method according to any one of claims 1 to 10, wherein in the first and/or second polypeptide, the amino acid at EU numbering position 397 is altered to Phe (F) or Tyr (Y).

12. The method according to any one of claims 1 to 11, wherein in the first polypeptide, the amino acid at EU numbering position 356 is altered to Lys (K), and the amino acid at EU numbering position 397 is altered to Phe (F) or Tyr (Y); and in the second polypeptide, the amino acid at EU numbering position 397 is altered to Phe (F) or Tyr (Y), and the amino acid at EU numbering position 439 is altered to Glu (E).

13. The method according to any one of claims 1 to 12, wherein the steps a) and b) are carried out by mixing a cell line producing the homo variant of the first polypeptides with a cell line producing the homo variant of the second polypeptides, and the step c) is carried out in the culture supernatant.

14. The method according to any one of claims 1 to 13, wherein the heteromultimer is a multispecific antibody or a hetero-Fc fusion protein.

15. The method according to any one of claims 1 to 14, wherein the heteromultimer is a bispecific antibody.

16. The method according to any one of claims 1 to 15, wherein the step c) described in claim 1 or claim 11 involves contact with a reducing agent.

17. The method according to claim 16, wherein the step c) involves the addition of an active substance selected from the group consisting of glutathione, L-cysteine, dithiothreitol, β-mercapto-ethanol, TCEP, and 2-MEA.

18. The method according to claim 17, wherein the step c) involves the addition of an active substance selected from glutathione and 2-MEA.

19. A bispecific antibody produced by a method according to any one of claims 1 to 18.

20. A composition comprising a bispecific antibody according to claim 19 and a pharmaceutically acceptable carrier.