DESCRIPTION

ANTIGEN-BINDING MOLECULE CAPABLE OF BINDING TO PLURALITY OF ANTIGEN MOLECULES REPEATEDLY

[Background Art]

Antibodies are drawing attention as pharmaceuticals as they are highly stable in plasma and have few side effects. At present, a number of IgG-type antibody pharmaceuticals are available on the market and many antibody pharmaceuticals are currently under development (Non-patent Documents 1 and 2). Meanwhile, various technologies applicable to second-generation antibody pharmaceuticals have been reported, including those that enhance effector function, antigen-binding ability, pharmacokinetics, and stability, and those that reduce the risk of immunogenicity (Non-patent Document 3). In general, the requisite dose of an antibody pharmaceutical is very high. This in turn has led to problems such as high production cost, as well as the difficulty in producing subcutaneous formulations. In theory, the dose of an antibody pharmaceutical may be reduced by improving antibody pharmacokinetics or improving the affinity between antibodies and antigens.

The literature has reported methods for improving antibody pharmacokinetics using artificial substitution of amino acids in constant regions (Non-patent Documents 4 and 5). Similarly, affinity maturation has been reported as a technology for enhancing antigen-binding ability or antigen-neutralizing activity (Non-patent Document 6). This technology enables enhancement of antigen-binding activity by introducing amino acid mutations into the CDR region of a variable region or such. The enhancement of antigen-binding ability enables improvement of *in vitro* biological activity or reduction of dosage, and further enables improvement of *in vivo* efficacy (Non-patent Document 7).

Meanwhile, the antigen-neutralizing capacity of a single antibody molecule depends on its affinity. By increasing the affinity, an antigen can be neutralized by a smaller amount of an antibody. Various methods can be used to enhance antibody affinity (Non-patent Document 6). Furthermore, if the affinity could be made infinite by covalently binding the antibody to the antigen, a single antibody molecule could neutralize one antigen molecule (a divalent antibody can neutralize two antigen molecules). However, the stoichiometric neutralization of one antibody against one antigen (one divalent antibody against two antigens) is the limit of pre-existing methods, and thus it was impossible to completely neutralize antigen with an amount of antibody smaller than the amount of antigen. In other words, the affinity-enhancing effect has a limit (Non-Patent Document 9). To prolong the neutralization effect of a neutralizing antibody

for a certain period, the antibody must be administered at a dose higher than the amount of antigen produced in the body during the same period. Therefore, with just the above-described improvement of antibody pharmacokinetics or affinity maturation technology, there were limitations when it comes to reduction of the required antibody dose. Accordingly, in order to sustain antibody's antigen-neutralizing effect for a target period with an amount of the antibody smaller than the amount of antigen, a single antibody must neutralize multiple antigens.

An antibody that binds to an antigen in a pH-dependent manner has recently been reported as a novel method for achieving the above objective (Patent Document 1). The antibodies with pH-dependent antigen binding, which strongly bind to an antigen under the neutral conditions in plasma and dissociate from the antigen under acidic conditions in the endosome, can dissociate from the antigen in the endosome. When an antibody with pH-dependent antigen binding dissociates from the antigen is recycled to the plasma by FcRn, it can bind to another antigen again. Thus, a single antibody can repeatedly bind to a number of antigens.

In addition, plasma retention of the antigen is very short as compared to antibodies recycled *via* FcRn binding. When an antibody with long plasma retention binds to such an antigen with a short plasma retention, the plasma retention time of the antigen-antibody complex is prolonged to the same as that of the antibody. Thus, the plasma retention of the antigen is prolonged by binding to the antibody, and thus the plasma antigen concentration is increased. In such cases, even if the antigen affinity of the antibody is improved, antigen elimination from the plasma cannot be enhanced. The above-described antibodies with pH-dependent antigen binding have been reported to be more effective as a method for enhancing antigen elimination from the plasma as compared to common antibodies (Patent Document 1).

Thus, a single antibody with pH-dependent antigen binding binds to a number of antigens and is capable of facilitating antigen elimination from the plasma as compared to common antibodies. Accordingly, the antibodies with pH-dependent antigen binding have effects not achieved by common antibodies. However, the only known method for achieving the effect of repeated binding of an antibody with pH-dependent antigen binding to antigen, and the effect of promoting antigen elimination from plasma, was to confer pH dependency on the antigen-antibody reaction using the pH difference between plasma and endosome.

Prior art documents related to the present invention are shown below:

Prior Art Documents

[Patent Documents]

[Patent Document 1] WO 2009/125825, ANTIGEN-BINDING MOLECULE CAPABLE OF BINDING TO TWO OR MORE ANTIGEN MOLECULES REPEATEDLY

[Non-patent Documents]

[Non-patent Document 1] Monoclonal antibody successes in the clinic, Janice M Reichert, Clark J Rosensweig, Laura B Faden & Matthew C Dewitz, Nature Biotechnology 23, 1073 - 1078 (2005)

[Non-patent Document 2] Pavlou AK, Belsey MJ., The therapeutic antibodies market to 2008., Eur J Pharm Biopharm. 2005 Apr; 59(3): 389-96

[Non-patent Document 3] Kim SJ, Park Y, Hong HJ., Antibody engineering for the development of therapeutic antibodies., Mol Cells. 2005 Aug 31; 20(1): 17-29. Review

[Non-patent Document 4] Hinton PR, Xiong JM, Johlfs MG, Tang MT, Keller S, Tsurushita N., An engineered human IgG1 antibody with longer serum half-life., J Immunol. 2006 Jan 1; 176(1): 346-56

[Non-patent Document 5] Ghetie V, Popov S, Borvak J, Radu C, Matesoi D, Medesan C, Ober RJ, Ward ES., Increasing the serum persistence of an IgG fragment by random mutagenesis., Nat Biotechnol. 1997 Jul; 15(7): 637-40

[Non-patent Document 6] Proc Natl Acad Sci U S A. 2005 Jun 14; 102(24): 8466-71. Epub 2005 Jun 6. A general method for greatly improving the affinity of antibodies by using combinatorial libraries. Rajpal A, Beyaz N, Haber L, Cappuccilli G, Yee H, Bhatt RR, Takeuchi T, Lerner RA, Crea R

[Non-patent Document 7] Wu H, Pfarr DS, Johnson S, Brewah YA, Woods RM, Patel NK, White WI, Young JF, Kiener PA. Development of Motavizumab, an Ultra-potent Antibody for the Prevention of Respiratory Syncytial Virus Infection in the Upper and Lower Respiratory Tract. J Mol Biol. (2007) 368: 652-665

[Non-patent Document 8] Hanson CV, Nishiyama Y, Paul S. Catalytic antibodies and their applications. Curr Opin Biotechnol. 2005 Dec; 16(6): 631-6

[Non-patent Document 9] Rathanaswami P, Roalstad S, Roskos L, Su QJ, Lackie S, Babcook J. Demonstration of an *in vivo* generated sub-picomolar affinity fully human monoclonal antibody to interleukin-8. Biochem Biophys Res Commun. 2005 Sep 9; 334(4): 1004-13

[Summary]

[Problems to be Solved]

The present disclosure was achieved in view of the above circumstances. An objective of the present disclosure is to provide methods for promoting antigen uptake into cells by using antigen-binding molecules, methods for increasing the number of times of antigen binding by one antigen-binding molecule, methods for promoting the reduction of plasma antigen concentration by administering antigen-binding molecules, methods for improving plasma

retention of antigen-binding molecules, antigen-binding molecules that facilitate antigen uptake into cells, antigen-binding molecules that have an increased number of times of antigen binding, antigen-binding molecules capable of promoting the reduction of plasma antigen concentration by administration, antigen-binding molecules with improved plasma retention, pharmaceutical compositions comprising the antigen-binding molecules, and methods for producing those described above.

[Means for Solving the Problems]

The present inventors conducted dedicated studies on methods for promoting antigen uptake into cells by antigen-binding molecules (molecules such as polypeptides having the antigen-binding activity), methods for increasing the number of times of antigen binding by one antigen-binding molecule, methods for promoting the reduction of plasma antigen concentration by administering antigen-binding molecules, and methods for improving the plasma retention of an antigen-binding molecule. As a result, the present inventors focused on the difference in the calcium concentration between plasma and early endosome, and then discovered that: antigen uptake into cells by antigen-binding molecules could he promoted by using antigen-binding molecules that have antigen-antibody reactivity in a calcium dependent manner; the number of times of antigen binding by one antigen-binding molecule could be increased by repetitive antigen binding of an antigen-binding molecule; the reduction of antigen concentration in plasma could be promoted by administering antigen-binding molecules; and that the plasma retention of antigen-binding molecule could be improved.

In one aspect, there is provided an antigen-binding molecule comprising an antigen-binding domain and a human FcRn-binding domain, whose antigen-binding activity is different under two different calcium concentration conditions and is lower under a low calcium concentration condition than under a high calcium concentration condition, and which has binding activity to human FcRn under a neutral pH condition.

In another aspect, there is provided an antigen-binding molecule comprising an antigen-binding domain and a human FcRn- binding domain, whose antigen-binding activity is different between two different calcium concentration conditions and is lower under a low calcium concentration condition than under a high calcium concentration condition, and wherein a light chain or heavy chain of the antigen-binding domain comprises a calcium-binding motif derived from a human antibody.

In another aspect, there is provided a method of producing an antigen-binding molecule having

at least one function selected from:

(i) function of promoting uptake of an antigen into cells,
(ii) function of binding to an antigen two or more times,
(iii) function of promoting the reduction of plasma antigen concentration, and
(iv) function of excellence in plasma retention,

wherein the method comprises the steps of (a) to (e) below:

(a) determining the antigen-binding activity of an antigen-binding molecule under a low calcium concentration condition;

(b) determining the antigen-binding activity of the antigen-binding molecule under a high calcium concentration condition;
(c) selecting an antigen-binding molecule that has a lower antigen-binding activity under the low calcium concentration condition than under the high calcium concentration condition;
(d) obtaining a gene encoding the antigen-binding molecule selected in step (c); and
(e) producing the antigen-binding molecule using the gene obtained in step (d).

In another aspect, there is provided a method of producing an antigen-binding molecule having at least one function selected from:

(i) function of promoting uptake of an antigen into cells,
(ii) function of binding to an antigen two or more times,
(iii) function of promoting the reduction of plasma antigen concentration, and
(iv) function of excellence in plasma retention,

wherein the method comprises the steps of (a) to (e) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a high calcium concentration condition;
(b) placing an antigen-binding molecule that binds to the antigen in step (a) under a low calcium concentration condition;
(c) obtaining an antigen-binding molecule that dissociates in step (b);
(d) obtaining a gene encoding the antigen-binding molecule obtained in step (c); and
(e) Producing the antigen-binding molecule using the gene obtained in step (d).

In another aspect, there is provided a method of producing an antigen-binding molecule having at least one function selected from:

(i) function of promoting uptake of an antigen into cells,
(ii) function of binding to an antigen two or more times,
(iii) function of promoting the reduction of plasma antigen concentration, and

(iv) function of excellence in plasma retention,

wherein the method comprises the steps of (a) to (f) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a low calcium concentration condition;

(b) selecting an antigen-binding molecule that does not bind to the antigen in step (a);

(c) allowing the antigen-binding molecule selected in step (b) to bind to the antigen under a high calcium concentration condition;

(d) obtaining an antigen-binding molecule that bound to the antigen in step (c);

(e) obtaining a gene encoding the antigen-binding molecule obtained in step (d); and

(f) producing the antigen-binding molecule using the gene obtained in step (e).

In another aspect, there is provided a pharmaceutical composition comprising: any one of the antigen-binding molecules described herein or an antigen-binding molecule produced by any one of the the production methods described herein, and a pharmaceutically acceptable carrier.

In another aspect, there is provided a pharmaceutical composition for use in promoting antigen uptake into cells or reduction of plasma antigen concentration, which comprises an antigen-binding molecule comprising an antigen-binding domain and a human FcRn-binding domain, whose antigen-binding activity is different between two different calcium concentrations and is lower under a low calcium concentration condition than under a high calcium concentration condition.

In another aspect, there is provided a method of screening for an antigen-binding molecule that has at least one function selected from:

(i) function of promoting uptake of an antigen into cells,

(ii) function of binding to an antigen two or more times

(iii) function of promoting the reduction of plasma antigen concentration, and

(iv) function of excellence in plasma retention,

wherein the method comprises the steps of (a) to (c) below:

(a) determining the antigen-binding activity of an antigen-binding molecule under a low calcium concentration condition;

(b) determining the antigen-binding activity of an antigen-binding molecule under a high calcium concentration condition; and

(c) selecting an antigen-binding molecule whose antigen-binding activity is lower under the

low calcium concentration condition than under the high calcium concentration condition.

In another aspect. there is provided a method of screening for an antigen-binding molecule that comprises at least one function selected from:

(i) function of promoting uptake of an antigen into cells,
(ii) function of binding to an antigen two or more times,
(iii) function of promoting the reduction of plasma antigen concentration, and
(iv) function of excellence in plasma retention,

wherein the method comprises the steps of (a) to (c) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a high calcium concentration condition;

(b) placing an antigen-binding molecule that binds to the antigen in step (a) under a low calcium concentration condition; and

(c) obtaining an antigen-binding molecule that dissociates in step (b).

In another aspect, there is provided a method of screening for an antigen-binding molecule that comprises at least one function selected from:

(i) function of promoting uptake of an antigen into cells,
(ii) function of binding to an antigen two or more times,
(iii) function of promoting the reduction of plasma antigen concentration, and
(iv) function of excellence in plasma retention,

wherein the method comprises the steps of (a) to (d) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a low calcium concentration condition;

(b) selecting an antigen-binding molecule that does not bind to the antigen in step (a);

(c) allowing the antigen-binding molecule selected in step (b) to bind to the antigen under a high calcium concentration condition; and

(d) obtaining an antigen-binding molecule bound to the antigen in step (c).

In another aspect, there is provided a method for increasing the number of times of antigen binding by one antigen-binding molecule by using any one of the antigen-binding molecules described herein or an antigen-binding molecule produced by any one of the production methods described herein.

In another aspect, there is provided a method for improving plasma retention of an antigen-binding molecule by using any one of the antigen-binding molecules described herein or an

antigen-binding molecule produced by any one of the production methods described herein.

In another aspect, there is provided a method for increasing the number of times of antigen binding by one antigen-binding molecule by using an antigen-binding molecule comprising an antigen-binding domain and a human FcRn-binding domain, whose antigen-binding activity is different between two different calcium concentrations and is lower under a low calcium concentration condition than under a high calcium concentration condition.

In another aspect, there is provided a method for improving plasma retention of an antigen-binding molecule by using an antigen-binding molecule comprising an antigen-binding domain and a human FcRn-binding domain, whose antigen-binding activity is different between two different calcium concentrations and is lower under a low calcium concentration condition than under a high calcium concentration condition.

Specifically, the present disclosure relates to methods for promoting antigen uptake into cells by using antigen-binding molecules that have antigen-antibody reactivity in a calcium dependent manner, methods for increasing the number of times of antigen binding by one antigen-binding molecule, methods for promoting the reduction of plasma antigen concentration by administering antigen-binding molecules, and methods for improving the plasma retention of antigen-binding molecules, as well as antigen-binding molecules that allow enhanced antigen uptake into cells, antigen-binding molecules with an increased number of times of antigen binding, antigen-binding molecules that can promote the reduction of plasma antigen concentration when administered, antigen-binding molecules with improved plasma retention, pharmaceutical compositions comprising the above antigen-binding molecules, and methods for producing them. More specifically, the present disclosure relates to the following: [1] an antigen-binding molecule comprising an antigen-binding domain and a human FcRn-binding domain, whose antigen-binding activity is different under two different calcium concentration conditions and is lower under a low calcium concentration condition than under a high calcium concentration condition, and which has binding activity to human FcRn under a

neutral pH condition;

[2] the antigen-binding molecule of [1], wherein the low calcium concentration is an ionized calcium concentration of 0.1 to 30 μM;

[3] the antigen-binding molecule of [1], wherein the high calcium concentration is an ionized calcium concentration of 100 μM to 10 mM;

[4] the antigen-binding molecule of [1] or [2], wherein the low calcium concentration is an intraendosomal concentration of ionized calcium;

[5] the antigen-binding molecule of [1] or [3], wherein the high calcium concentration is a plasma concentration of ionized calcium;

[6] the antigen-binding molecule of any of [1] to [5], wherein the FcRn-binding domain is an Fc region;

[7] the antigen-binding molecule of any of [1] to [6], further wherein the antigen-binding activity is lower under an acidic pH condition than under a neutral pH condition;

[8] the antigen-binding molecule of [7], wherein at least one amino acid is substituted with histidine, or at least one histidine is inserted into the antigen-binding molecule;

[9] the antigen-binding molecule of any of [1] to [8], which binds to a membrane antigen or soluble antigen;

[10] the antigen-binding molecule of any of [1] to [9], wherein the antigen is an antigen selected from the group consisting of IL-6R, IL-6, IgA, human glypican 3, and IgE;

[11] an antigen-binding molecule comprising an antigen-binding domain and a human FcRn-binding domain, whose antigen-binding activity is different between two different calcium concentration conditions and is lower under a low calcium concentration condition than under a high calcium concentration condition, and wherein a light chain or heavy chain of the antigen-binding domain comprises a calcium-binding motif derived from a human antibody;

[12] the antigen-binding molecule of [11], wherein the calcium-binding motif is comprised in the light chain CDR1, CDR2, and/or CDR3 of the antigen-binding domain;

[13] the antigen-binding molecule of [12], wherein the calcium-binding motif is comprised at positions 30, 31, and/or 32 according to Kabat's numbering in the light chain CDR1;

[14] the antigen-binding molecule of [12] or [13], wherein the calcium-binding motif is comprised at position 50 according to Kabat's numbering in the light chain CDR2;

[15] the antigen-binding molecule of any of [12] to [14], wherein the calcium-binding motif is comprised at position 92 according to Kabat's numbering in the light chain CDR3;

[16] the antigen-binding molecule of any of [12] to [15], which is either IgA or human glypican 3;

[17] the antigen-binding molecule of [11], wherein the calcium-binding motif is comprised in the heavy chain CDR1, CDR2, and/or CDR3 of the antigen-binding domain;

[18] the antigen-binding molecule of [16], wherein the calcium-binding motif is comprised at positions 95, 96, 100a, and/or 101 according to Kabat's numbering in the heavy chain CDR3;
[19] the antigen-binding molecule of [17] or [18], which is either IL-6R or IL-6;
[20] the antigen-binding molecule of any of [11] to [19], which comprises an FcRn-binding domain that has FcRn-binding activity in the neutral pH range;
[21] the antigen-binding molecule of [20], wherein the FcRn-binding domain is an Fc region;
[22] the antigen-binding molecule of any of [1] to [10], [20], or [21], wherein one or more amino acids at positions 248, 250, 252, 254, 255, 256, 257, 258, 265, 286, 289, 297, 303, 305, 307, 308, 309, 311, 312, 314, 315, 317, 332, 334, 360, 376, 380, 382, 384, 385, 386, 387, 389, 424, 428, 433, 434, and 436 (EU numbering) in the amino acid sequence of the Fc region are different from those of the natural Fc region;
[23] the antigen-binding molecule of [22], which comprises any one or combination of:
Met at amino acid position 237;
Ile at amino acid position 248;
Ala, Phe, Ile, Met, Gln, Ser, Val, Trp, or Tyr at amino acid position 250;
Phe, Trp, or Tyr at amino acid position 252;
Thr at amino acid position 254;
Glu at amino acid position 255;
Asp, Glu, or Gln at amino acid position 256;
Ala, Gly, Ile, Leu, Met, Asn, Ser, Thr, or Val at amino acid position 257;
His at amino acid position 258;
Ala at amino acid position 265;
Ala or Glu at amino acid position 286;
His at amino acid position 289;
Ala at amino acid position 297;
Ala at amino acid position 303;
Ala at amino acid position 305;
Ala, Asp, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Val, Trp, or Tyr at amino acid position 307;
Ala, Phe, Ile, Leu, Met, Pro, Gln, or Thr at amino acid position 308;
Ala, Asp, Glu, Pro, or Arg at amino acid position 309;
Ala, His, or Ile at amino acid position 311;
Ala or His at amino acid position 312;
Lys or Arg at amino acid position 314;
Ala, Asp, or His at amino acid position 315;
Ala at amino acid position 317;

Val at amino acid position 332;
Leu at amino acid position 334;
His at amino acid position 360;
Ala at amino acid position 376;
Ala at amino acid position 380;
Ala at amino acid position 382;
Ala at amino acid position 384;
Asp or His at amino acid position 385;
Pro at amino acid position 386;
Glu at amino acid position 387;
Ala or Ser at amino acid position 389;
Ala at amino acid position 424;
Ala, Asp, Phe, Gly, His, Ile, Lys, Leu, Asn, Pro, Gln, Ser, Thr, Val, Trp, or Tyr at amino acid position 428;
Lys at amino acid position 433;
Ala, Phe, His, Ser, Trp, or Tyr at amino acid position 434; or
His, Ile, Leu, or Val at amino acid position 436;
according to EU numbering in the Fc region;

[24] the antigen-binding molecule of any of [1] to [23], wherein the antigen-binding molecule is an antibody;

[25] a method of producing an antigen-binding molecule having at least one function selected from:

(i) function of promoting uptake of an antigen into cells,
(ii) function of binding to an antigen two or more times,
(iii) function of promoting the reduction of plasma antigen concentration, and
(iv) function of excellence in plasma retention,

wherein the method comprises the steps of (a) to (e) below:

(a) determining the antigen-binding activity of an antigen-binding molecule under a low calcium concentration condition;
(b) determining the antigen-binding activity of the antigen-binding molecule under a high calcium concentration condition;
(c) selecting an antigen-binding molecule that has a lower antigen-binding activity under the low calcium concentration condition than under the high calcium concentration condition;
(d) obtaining a gene encoding the antigen-binding molecule selected in step (c); and
(e) producing the antigen-binding molecule using the gene obtained in step (d);

[26] a method of producing an antigen-binding molecule having at least one function selected from:

(i) function of promoting uptake of an antigen into cells,

(ii) function of binding to an antigen two or more times,

(iii) function of promoting the reduction of plasma antigen concentration, and

(iv) function of excellence in plasma retention,

wherein the method comprises the steps of (a) to (e) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a high calcium concentration condition;

(b) placing an antigen-binding molecule that binds to the antigen in step (a) under a low calcium concentration condition;

(c) obtaining an antigen-binding molecule that dissociates in step (b);

(d) obtaining a gene encoding the antigen-binding molecule obtained in step (c); and

(e) producing the antigen-binding molecule using the gene obtained in step (d);

[27] a method of producing an antigen-binding molecule having at least one function selected from:

(i) function of promoting uptake of an antigen into cells,

(ii) function of binding to an antigen two or more times,

(iii) function of promoting the reduction of plasma antigen concentration, and

(iv) function of excellence in plasma retention,

wherein the method comprises the steps of (a) to (f) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a low calcium concentration condition;

(b) selecting an antigen-binding molecule that does not bind to the antigen in step (a);

(c) allowing the antigen-binding molecule selected in step (b) to bind to the antigen under a high calcium concentration condition;

(d) obtaining an antigen-binding molecule that bound to the antigen in step (c);

(e) obtaining a gene encoding the antigen-binding molecule obtained in step (d); and

(f) producing the antigen-binding molecule using the gene obtained in step (e);

[28] the production method of any of [25] to [27], which additionally comprises the step of conferring or increasing the human FcRn-binding activity under a neutral pH condition by modifying an amino acid in the antigen-binding molecule;

[29] the production method of any of [25] to [27], which additionally comprises the step of reducing the antigen-binding activity under an acidic pH condition to be lower than that under a neutral pH condition by modifying an amino acid in the antigen-binding molecule;

[30] the production method of any one of [25] to [27], wherein the low calcium concentration is

an ionized calcium concentration of 0.1 to 30 μM;

[31] the production method of any of [25] to [27], wherein the high calcium concentration is an ionized calcium concentration of 100 μM to 10 mM;

[32] the production method of any of [25] to [27], wherein the low calcium concentration is an intraendosomal concentration of ionized calcium;

[33] the production method of any of [25] to [27], wherein the high calcium concentration is a plasma concentration of ionized calcium;

[34] the production method of [29], wherein the amino acid modification in the antigen-binding molecule is modification by substituting at least one amino acid with histidine, or inserting at least one histidine into the antigen-binding molecule;

[35] the production method of any of [25] to [34], wherein an antigen bound by the antigen-binding molecule is an antigen selected from the group consisting of IL-6R, IL-6, IgA, human glypican 3, and IgE;

[36] the production method of any of [25] to [35], wherein the antigen-binding molecule is an antibody;

[37] a pharmaceutical composition comprising:
the antigen-binding molecule of any of [1] to [24] or an antigen-binding molecule produced by the production method of any of [25] to [36], and a pharmaceutically acceptable carrier;

[38] the pharmaceutical composition of [37] for use in promoting internalization of the antigen into cells;

[39] the pharmaceutical composition of [37] for use in promoting reduction of the antigen concentration in plasma;

[40] a pharmaceutical composition for use in promoting antigen uptake into cells or reduction of plasma antigen concentration, which comprises an antigen-binding molecule comprising an antigen-binding domain and a human FcRn-binding domain, whose antigen-binding activity is different between two different calcium concentrations and is lower under a low calcium concentration condition than under a high calcium concentration condition;

[41] the pharmaceutical composition of [40], wherein the low calcium concentration is an ionized calcium concentration of 0.1 to 30 μM;

[42] the pharmaceutical composition of [40], wherein the high calcium concentration is an ionized calcium concentration of 100 μM to 10 mM;

[43] the pharmaceutical composition of [40] or [41], wherein the low calcium concentration is an intraendosomal concentration of ionized calcium;

[44] the pharmaceutical composition of [40] or [42], wherein the high calcium concentration is a plasma concentration of ionized calcium;

[45] the pharmaceutical composition of any of [40] to [44], wherein the FcRn-binding domain

comprised in the antigen-binding molecule is an Fc region;

[46] the pharmaceutical composition of any of [40] to [45], wherein the antigen-binding activity of the antigen-binding molecule is lower under an acidic pH condition than under a neutral pH condition;

[47] the pharmaceutical composition of [46], wherein the amino acid modification in the antigen-binding molecule is modification by substituting at least one amino acid with histidine, or inserting at least one histidine into the antigen-binding molecule;

[48] the pharmaceutical composition of any of [40] to [47], wherein the antigen to which the antigen-binding molecule binds is an antigen selected from the group consisting of IL-6R, IL-6, IgA, human glypican 3, and IgE;

[49] a method of screening for an antigen-binding molecule that has at least one function selected from:

(i) function of promoting uptake of an antigen into cells,

(ii) function of binding to an antigen two or more times,

(iii) function of promoting the reduction of plasma antigen concentration, and

(iv) function of excellence in plasma retention,

wherein the method comprises the steps of (a) to (c) below:

(a) determining the antigen-binding activity of an antigen-binding molecule under a low calcium concentration condition;

(b) determining the antigen-binding activity of an antigen-binding molecule under a high calcium concentration condition; and

(c) selecting an antigen-binding molecule whose antigen-binding activity is lower under the low calcium concentration condition than under the high calcium concentration condition;

[50] a method of screening for an antigen-binding molecule that comprises at least one function selected from:

(i) function of promoting uptake of an antigen into cells,

(ii) function of binding to an antigen two or more times,

(iii) function of promoting the reduction of plasma antigen concentration, and

(iv) function of excellence in plasma retention,

wherein the method comprises the steps of (a) to (c) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a high calcium concentration condition;

(b) placing an antigen-binding molecule that binds to the antigen in step (a) under a low calcium concentration condition; and

(c) obtaining an antigen-binding molecule that dissociates in step (b);

[51] a method of screening for an antigen-binding molecule that comprises at least one function selected from:

(i) function of promoting uptake of an antigen into cells,

(ii) function of binding to an antigen two or more times,

(iii) function of promoting the reduction of plasma antigen concentration, and

(iv) function of excellence in plasma retention,

wherein the method comprises the steps of (a) to (d) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a low calcium concentration condition;

(b) selecting an antigen-binding molecule that does not bind to the antigen in step (a);

(c) allowing the antigen-binding molecule selected in step (b) to bind to the antigen under a high calcium concentration condition; and

(d) obtaining an antigen-binding molecule bound to the antigen in step (c);

[52] the screening method of any of [49] to [51], wherein the low calcium concentration is an ionized calcium concentration of 0.1 to 30 μM;

[53] the screening method of any of [49] to [51], wherein the high calcium concentration is an ionized calcium concentration of 100 μM to 10 mM;

[54] the screening method of any of [49] to [52], wherein the low calcium concentration is an intraendosomal concentration of ionized calcium;

[55] the screening method of any of [49] to [51], or [53], wherein the high calcium concentration is a plasma concentration of ionized calcium;

[56] the screening method of any of [49] to [55], wherein the antigen to which the antigen-binding molecule binds is an antigen selected from the group consisting of IL-6R, IL-6, IgA, human glypican 3, and IgE;

[57] the screening method of any of [49] to [56], wherein the antigen-binding molecule is an antibody;

[58] a method for promoting antigen uptake into a cell by an antigen-binding molecule by administering the antigen-binding molecule of any of [1] to [24] or an antigen-binding molecule produced by the production method of any of [25] to [36];

[59] a method for promoting the reduction of plasma antigen concentration by administering the antigen-binding molecule of any of [1] to [24] or an antigen-binding molecule produced by the production method of any of [25] to [36];

[60] a method for increasing the number of times of antigen binding by one antigen-binding molecule by using the antigen-binding molecule of any of [1] to [24] or an antigen-binding molecule produced by the production method of any of [25] to [36];

[61] a method for improving plasma retention of an antigen-binding molecule by using the

antigen-binding molecule of any of [1] to [24] or an antigen-binding molecule produced by the production method of any of [25] to [36];

[62] a method for promoting antigen uptake into a cell by an antigen-binding molecule by administering an antigen-binding molecule comprising an antigen-binding domain and a human FcRn-binding domain, whose antigen-binding activity is different between two different calcium concentrations and is lower under a low calcium concentration condition than under a high calcium concentration condition;

[63] a method for promoting the reduction of plasma antigen concentration by administering an antigen-binding molecule comprising an antigen-binding domain and a human FcRn-binding domain, whose antigen-binding activity is different between two different calcium concentrations and is lower under a low calcium concentration condition than under a high calcium concentration condition;

[64] a method for increasing the number of times of antigen binding by one antigen-binding molecule by using an antigen-binding molecule comprising an antigen-binding domain and a human FcRn-binding domain, whose antigen-binding activity is different between two different calcium concentrations and is lower under a low calcium concentration condition than under a high calcium concentration condition;

[65] a method for improving plasma retention of an antigen-binding molecule by using an antigen-binding molecule comprising an antigen-binding domain and a human FcRn-binding domain, whose antigen-binding activity is different between two different calcium concentrations and is lower under a low calcium concentration condition than under a high calcium concentration condition;

[66] the method of any of [62] to [65], wherein the low calcium concentration is an ionized calcium concentration of 0.1 to 30 μM;

[67] the method of any of [62] to [66], wherein the high calcium concentration is an ionized calcium concentration of 100 μM to 10 mM;

[68] the method of any of [62] to [67], wherein the low calcium concentration is an intraendosomal concentration of ionized calcium;

[69] the method of any of [62] to [68], wherein the high calcium concentration is a plasma concentration of ionized calcium;

[70] the method of any of [62] to [69], wherein an FcRn-binding domain of the antigen-binding molecule is an Fc region;

[71] the method of any of [62] to [70], wherein additionally the antigen-binding activity of the antigen-binding molecule is lower under an acidic pH condition than under a neutral pH condition;

[72] the method of [71], wherein the amino acid modification in the antigen-binding molecule is

modification by substituting at least one amino acid with histidine, or inserting at least one histidine into the antigen-binding molecule;

[73] the method of any of [62] to [72], wherein the antigen to which the antigen-binding molecule binds is an antigen selected from the group consisting of IL-6R, IL-6, IgA, human glypican 3, and IgE; and

[74] the method of any of [62] to [73], wherein the antigen-binding molecule is an antibody.

Furthermore, the present disclosure relates to kits for use in the methods of the present disclosure, which comprise an antigen-binding molecule of the present disclosure or an antigen-binding molecule produced by production methods of the present disclosure. The present disclosure also relates to agents for promoting antigen uptake into cells by an antigen-binding molecule, agents for promoting the reduction of plasma antigen concentration, agents for increasing the number of times of antigen binding by one antigen-binding molecule, and agents for improving plasma retention of an antigen-binding molecule, all of which comprise as an active ingredient an antigen-binding molecule of the present disclosure or an antigen-binding molecule produced by the production method of the present disclosure. Furthermore, the present disclosure relates to the use of an antigen-binding molecule of the present disclosure or an antigen-binding molecule produced by the production methods of the present disclosure in the production of agents for promoting antigen uptake into cells by an antigen-binding molecule, agents for promoting reduction of plasma antigen concentration, agents for increasing the number of times of antigen binding by an antigen-binding molecule, or agents for improving plasma retention of an antigen-binding molecule. The present disclosure also relates to antigen-binding molecules of the present disclosure or antigen-binding molecules produced by production methods of the present disclosure for use in the methods of the present disclosure.

[Effects of the Disclosure]

The present disclosure provides methods for promoting antigen uptake into cells by antigen-binding molecules, methods for increasing the number of times of antigen binding by one antigen-binding molecule, methods for promoting the reduction of plasma antigen concentration by administering antigen-binding molecules, and methods for improving the plasma retention of an antigen-binding molecule. Promotion of antigen uptake into cells by antigen-binding molecules enables one to promote reduction of plasma antigen concentration by administering the antigen-binding molecules and also to promote the plasma retention of an antigen-binding molecule. This can increase the number of times of antigen binding by one antigen-binding molecule. Thus, such antigen-binding molecules can produce more superior *in vivo* effects as compared to typical antigen-binding molecules.

[Brief Description of the Drawings]

Fig. 1 is a diagram showing that an antibody with pH-dependent binding repeatedly binds to soluble antigens. (i) an antibody binds to soluble antigens; (ii) the antibody is non-specifically internalized into a cell *via* pinocytosis; (iii) the antibody binds to FcRn within the endosome, and then the soluble antigens dissociate from the antibody; (iv) the soluble antigens are transferred into the lysosome and degraded; (v) after dissociation from the soluble antigens, the antibody is recycled to the plasma *via* FcRn; (vi) the recycled antibody can bind to soluble antigens again.

Fig. 2 is a diagram showing that an antibody with pH-dependent binding repeatedly binds to membrane antigens. (i) an antibody binds to membrane antigens; (ii) the antibody is internalized into a cell in a complex with the membrane antigens; (iii) the antibody dissociates from the membrane antigens within the endosome; (iv) the membrane antigens are transferred into the lysosome and degraded; (v) after dissociation from the membrane antigens, the antibody is recycled to the plasma; (vi) the recycled can bind to membrane antigens again.

Fig. 3 is a diagram showing the modes of interaction in plasma (pH 7.4) and endosome (pH 6.0) between an antigen and an antibody with pH-dependent binding.

Fig. 4 is a diagram showing the modes of interaction in plasma (Ca^{2+} 2 mM) and endosome (Ca^{2+} 3 μM) between an antigen and an antibody with calcium-dependent binding.

Fig. 5 is a diagram showing the modes of interaction in plasma (pH 7.4, Ca^{2+} 2 mM) and endosome (pH 6.0, Ca^{2+} 3 μM) between an antigen and an antibody with pH- and calcium-dependent binding.

Fig. 6 presents Biacore sensorgrams showing the interaction of anti-human IL-6 receptor antibodies with soluble human IL-6 receptor under the conditions of (Ca^{2+}2 mM) and (Ca^{2+} 3 μM).

Fig. 7 presents a Biacore sensorgram showing the interaction of H54/L28-IgG1 with soluble human IL-6 receptor under the conditions of (Ca^{2+} 2 mM) and (Ca^{2+} 3 μM).

Fig. 8 presents a Biacore sensorgram showing the interaction of FH4-IgG1 with soluble human IL-6 receptor under the conditions of (Ca^{2+} 2 mM) and (Ca^{2+} 3 μM).

Fig. 9 presents a Biacore sensorgram showing the interaction of 6RL#9-IgG1 with soluble human IL-6 receptor under the conditions of (Ca^{2+} 2 mM) and (Ca^{2+} 3 μM).

Fig. 10 describes a time course of the plasma antibody concentration in normal mice administered with H54/L28-IgG1, FH4-IgG1, or 6RL#9-IgG1.

Fig. 11 describes a time course of the plasma level of soluble human IL-6 receptor (hsIL-6R) in normal mice administered with H54/L28-IgG1, FH4-IgG1, or 6RL#9-IgG1.

Fig. 12 describes a time course of the plasma antibody concentration in normal mice administered with H54/L28-N434W, FH4-N434W, or 6RL#9-N434W.

Fig. 13 describes a time course of the plasma level of soluble human IL-6 receptor (hsIL-6R) in normal mice administered with H54/L28-N434W, FH4-N434W, or 6RL#9-N434W.

Fig. 14 shows the structure of heavy-chain CDR3 of an Fab fragment from antibody 6RL#9 determined by X-ray crystallography.

Fig. 15 presents Biacore sensorgrams showing the interaction of anti-human IL-6 antibodies with human IL-6 under the conditions of (Ca^{2+} 1.2 mM) and (Ca^{2+} 3 μM).

Fig. 16 shows ion-exchange chromatograms for an antibody having human Vk5-2 sequence and an antibody having h Vk5-2_L65 sequence which has a modified glycosylation sequence in the human Vk5-2 sequence. Solid line indicates a chromatogram for an antibody having human Vk5-2 sequence (heavy chain: CIM_H, SEQ ID NO: 48; light chain: hVk5-2, fusion molecule between SEQ ID NOs: 41 and 28); broken line indicates a chromatogram for an antibody having hVk5-2_L65 sequence (heavy chain: CIM_H (SEQ ID NO: 48); light chain: hVk5-2_L65 (SEQ ID NO: 47)).

Fig. 17 shows ion-exchange chromatograms for an antibody having LfVk1_Ca sequence (heavy chain: GC_H, SEQ ID NO: 102; light chain: LfVk1_Ca, SEQ ID NO: 61) and an antibody having a sequence in which Asp (D) in the LfVk1_Ca sequence is substituted with Ala (A) after storage at 5°C (solid line) or 50°C (dotted line). After storage at 5°C, the highest peak in the chromatogram for each antibody is defined as a main peak, and the y axis of each ion-exchange chromatogram was normalized to the main peak.

Fig. 18 shows ion-exchange chromatograms for an antibody having LfVk1_Ca sequence (heavy chain: GC_H, SEQ ID NO: 102; light chain: LfVk1_Ca, SEQ ID NO: 61) and an antibody having LfVk1_Ca6 sequence (heavy chain: GC_H, SEQ ID NO: 102; light chain: LfVk1_Ca6, SEQ ID NO: 75) in which Asp (D) at position 30 (Kabat's numbering system) in the LfVk1_Ca sequence is substituted with Ser (S) after storage at 5°C (solid line) or 50°C (dotted line). After storage at 5°C, the highest peak in the chromatogram for each antibody is defined as a main peak, and the y axis of each ion-exchange chromatogram was normalized to the main peak.

Fig. 19 presents Biacore sensorgrams showing the interaction of anti-human CD4 antibodies with soluble human CD4 under the conditions of (Ca^{2+} 1.2 mM) and (Ca^{2+} 3 μM).

Fig. 20 describes a time course of the plasma concentration of anti-human CD4 antibodies in normal mice.

Fig. 21 describes a time course of the plasma concentration of soluble human CD4 in the group administered with soluble human CD4 alone, the antibody TNX355-IgG1-administered group, the antibody Q425-administered group, and the antibody Q425L9-administered group of normal mice.

Fig. 22 presents Biacore sensorgrams showing the interaction of anti-human IgA

antibodies with human IgA under the conditions of (Ca^{2+} 1.2 mM) and (Ca^{2+} 3 μM).

Fig. 23 describes a time course of plasma antibody concentrations in normal mice for the antibody GA1-IgG1-administered group, the antibody GA2-IgG1-administered group, the antibody GA3-IgG1-administered group, and the GA2-N434W-administered group.

Fig. 24 describes a time course of the plasma human IgA concentration in normal mice for the group administered with human IgA alone, the antibody GA1-IgG1-administered group, the antibody GA2-IgG1-administered group, the antibody GA3-IgG1-administered group, and the antibody GA2-N434W-administered group.

Fig. 25 describes a time course of the plasma concentration of unbound human IgA in normal mice for the antibody GA1-IgG1-administered group, the antibody GA2-IgG1-administered group, the antibody GA3-IgG1-administered group, and the antibody GA2-N434W-administered group.

Fig. 26 is an illustrative diagram showing the efficiency of antigen elimination per antibody molecule for a general antibody that forms a large immune complex with a multimeric antigen.

Fig. 27 is an illustrative diagram showing the efficiency of antigen elimination per antibody molecule for a pH/Ca-dependent antibody having the constant region of natural IgG1 which forms a large immune complex with a multimeric antigen.

Fig. 28 is an illustrative diagram showing the efficiency of antigen elimination per antibody molecule for a pH/Ca-dependent multispecific antibody that recognizes two or more epitopes in a monomeric antigen and is suitable for formation of a large immune complex.

Fig. 29 describes the interaction of anti-human glypican 3 antibodies with recombinant human glypican 3 under the conditions of (Ca^{2+} 1.2 mM) and (Ca^{2+} 3 μM) by ELISA.

Fig. 30 describes the interaction of anti-human IgE antibodies with recombinant human IgE under the conditions of (Ca^{2+} 2 mM) and (Ca^{2+} 3 μM) by ELISA.

Fig. 31 describes a time course of plasma antibody concentrations in human FcRn transgenic mice.

Fig. 32 describes a time course of the plasma concentration of soluble human IL-6 receptor in human FcRn transgenic mice.

Fig. 33 describes a time course of plasma antibody concentrations in normal mice.

Fig. 34 describes a time course of the plasma concentration of soluble human IL-6 receptor in normal mice.

Fig. 35 describes a time course of the plasma concentration of unbound soluble human IL-6 receptor in normal mice.

Fig. 36 describes a time course of the plasma concentration of soluble human IL-6 receptor in human FcRn transgenic mice.

Fig. 37 describes a time course of the plasma concentration of soluble human IL-6 receptor after administration of Fv4-IgG1-F14 at a lower dose (0.01 mg/kg) or 1 mg/kg.

Fig. 38 describes a time course of plasma antibody concentrations after administration of Fv4-IgG1-F14 at a lower dose (0.01 mg/kg) or 1 mg/kg.

Fig. 39 describes a time course of the plasma concentration of soluble human IL-6 receptor after administration of anti-human IL-6 receptor antibodies to normal mice in which the plasma concentration of soluble human IL-6 receptor is constant.

Fig. 40 describes a time course of plasma antibody concentration after co-administration of hsIL-6R and an anti-human IL-6 receptor antibody to human FcRn transgenic mice (lineage 276).

Fig. 41 describes a time course of the plasma concentration of soluble human IL-6 receptor after co-administration of hsIL-6R and an anti-human IL-6 receptor antibody to human FcRn transgenic mice (lineage 276).

Fig. 42 describes the relationship between the binding affinity of Fc variants to human FcRn at pH 7.0 and plasma hsIL-6R concentration one day after co-administration of hsIL-6R and an anti-human IL-6 receptor antibody to human FcRn transgenic mice (lineage 276).

Fig. 43 describes the relationship between the binding affinity of Fc variants to human FcRn at pH 7.0 and plasma antibody concentration one day after co-administration of hsIL-6R and an anti-human IL-6 receptor antibody to human FcRn transgenic mice (lineage 276).

Fig. 44 describes a time course of the molar antigen/antibody ratio (C value) after co-administration of hsIL-6R and an anti-human IL-6 receptor antibody to human FcRn transgenic mice (lineage 276).

Fig. 45 describes the relationship between the binding affinity of Fc variants to human FcRn at pH 7.0 and the molar antigen/antibody ratio (C value) at day 1 after co-administration of hsIL-6R and an anti-human IL-6 receptor antibody to human FcRn transgenic mice (lineage 276).

Fig. 46 describes a time course of the plasma concentration of hsIL-6R after administration of Fv4-IgG1-F14 at lower doses (0.01 or 0.2 mg/kg) or 1 mg/kg to human FcRn transgenic mice (lineage 276) in which the plasma concentration of hsIL-6R is constant (steady-state infusion model).

Fig. 47 describes a time course of the plasma hsIL-6R concentration in human FcRn transgenic mouse lineage 276 and lineage 32 after co-administration of hsIL-6R and anti-human IL-6 receptor antibody to human FcRn transgenic mice (lineages 276 and 32).

Fig. 48 describes a time course of plasma antibody concentration in human FcRn transgenic mouse lineage 276 and lineage 32 after co-administration of hsIL-6R and anti-human IL-6 receptor antibody to human FcRn transgenic mice (lineages 276 and 32).

Fig. 49 describes a time course of the plasma concentration of hsIL-6R after administration of anti-human IL-6 receptor antibody to human FcRn transgenic mice in which the plasma concentration of hsIL-6R is constant (lineage 32) (steady-state infusion model).

Fig. 50 describes a time course of plasma antibody concentration after administration of anti-human IL-6 receptor antibody to human FcRn transgenic mice in which the plasma concentration of hsIL-6R is constant (lineage 32) (steady-state infusion model).

Fig 51 describes time courses of the molar antigen/antibody ratio (value C) after administration of anti-human IL-6 receptor antibody to human FcRn transgenic mice in which the plasma concentration of hsIL-6R is constant (lineage 32) (steady-state infusion model).

Fig. 52 describes the relationship between the binding affinity of Fc variants to human FcRn at pH 7.0 and molar antigen/antibody ratio (value C) at day 1 after administration of anti-human IL-6 receptor antibody to human FcRn transgenic mice (lineage 32) in which the plasma concentration of hsIL-6R is constant (steady-state infusion model).

Fig. 53 shows in a graph a time course of plasma antibody concentration after administration of anti-human IL-6 receptor antibodies having Fc variant of F11, F39, F48, and F264 to human FcRn transgenic mice in which the plasma concentration of hsIL-6R is constant (lineage 32) (steady-state infusion model).

Fig. 54 shows in a graph a time course of the plasma concentration of hsIL-6R after administration of anti-human IL-6 receptor antibodies having Fc variant of F11, F39, F48, and F264 to human FcRn transgenic mice in which the plasma concentration of hsIL-6R is constant (lineage 32) (steady-state infusion model).

Fig. 55 describes a time course of plasma antibody concentration after administration of anti-human IL-6 receptor antibodies having Fc variant of F157, F196, and F262 to human FcRn transgenic mice in which the plasma concentration of hsIL-6R is constant (lineage 32) (steady-state infusion model).

Fig. 56 describes a time course of the plasma concentration of hsIL-6R after administration of anti-human IL-6 receptor antibodies having Fc variant of F157, F196, and F262 to human FcRn transgenic mice in which the plasma concentration of hsIL-6R is constant (lineage 32) (steady-state infusion model).

[Mode for Carrying Out the Invention]

The present invention provides methods for promoting antigen uptake into cells by antigen-binding molecules, methods for increasing the number of times of antigen binding by one antigen-binding molecule, methods for promoting the reduction of plasma antigen concentration by administering antigen-binding molecules, and methods for improving the plasma retention of an antigen-binding molecule. Specifically, the present invention provides

methods for promoting antigen uptake into cells by antigen-binding molecules, methods for increasing the number of times of antigen binding by one antigen-binding molecule, methods for promoting the reduction of plasma antigen concentration by administering antigen-binding molecules, and methods for improving the plasma retention of antigen-binding molecules, all of which use an antigen-binding molecule that has a lower antigen-binding activity (herein, sometimes referred to as "binding activity") under a low calcium concentration condition than under a high calcium concentration condition.

Amino acids

Herein, amino acids are described in one- or three-letter codes or both, for example, Ala/A, Leu/L, Arg/R, Lys/K, Asn/N, Met/M, Asp/D, Phe/F, Cys/C, Pro/P, Gln/Q, Ser/S, Glu/E, Thr/T, Gly/G, Trp/W, His/H, Tyr/Y, Ile/I, or Val/V.

Antigens

Herein, "antigens" are not particularly limited in their structure, as long as they comprise epitopes to which antigen-binding domains bind. In other words, antigens can be inorganic or organic substances; and alternatively, antigens can be foreign or endogenous substances to organisms subjected to the administration of the present invention. Examples of antigens bound by the antigen-binding domains of antigen-binding molecules whose pharmacokinetics is improved by methods of the present invention preferably include membrane antigens such as receptor proteins (membrane-bound receptors and soluble receptors) and cell surface markers; soluble antigens such as cytokines; and antigens with epitopes present only in foreign organisms. Such antigens include, for example, the following molecules: 17-IA, 4-1 BB, 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, Addressins, adiponectin, ADP ribosyl cyclase-1, aFGF, AGE, ALCAM, ALK, ALK-1, ALK-7, allergen, α1-anticymotrypsin, α1-antitrypsin, α-synuclein, α-V/β-1 antagonist, aminin, amylin, amyloid β, amyloid immunoglobulin heavy-chain variable region, amyloid immunoglobulin light-chain variable region, Androgen, ANG, angiotensinogen, Angiopoietin ligand-2, anti-Id, antithrombinIII, Anthrax, APAF-1, APE, APJ, apo A1, apo serum amyloid A, Apo-SAA, APP, APRIL, AR, ARC, ART, Artemin, ASPARTIC, Atrial natriuretic factor, Atrial natriuretic peptide, atrial natriuretic peptides A, atrial natriuretic peptides B, atrial natriuretic peptides C, av/b3 integrin, Axl, B7-1, B7-2, B7-H, BACE, BACE-1, Bacillus anthracis protective antigen, Bad, BAFF, BAFF-R, Bag-1, BAK, Bax, BCA-1, BCAM, Bcl, BCMA, BDNF, b-ECGF, β-2-

microglobulin, β lactamase, bFGF, BID, Bik, BIM, BLC, BL-CAM, BLK, B-lymphocyte Stimulator (BlyS), 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), BMPR, BMPR-IA (ALK-3), BMPR-IB (ALK-6), BMPR-II (BRK-3), BMPs, BOK, Bombesin, Bone-derived neurotrophic factor, bovine growth hormone, BPDE, BPDE-DNA, BRK-2, BTC, B-lymphocyte cell adhesion molecule, C10, C1-inhibitor, C1q, C3, C3a, C4, C5, C5a (complement 5a), CA125, CAD-8, Cadherin-3, Calcitonin, cAMP, Carbonic anhydrase-IX, carcinoembryonic antigen (CEA), carcinoma-associated antigen, Cardiotrophin-1, 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/I-309, CCL11/Eotaxin, CCL12/MCP-5, CCL13/MCP-4, CCL14/HCC-1, CCL15/HCC-2, CCL16/HCC-4, CCL17/TARC, CCL18/PARC, CCL19/ELC, CCL2/MCP-1, CCL20/MIP-3-α, CCL21/SLC, CCL22/MDC, CCL23/MPIF-1, CCL24/Eotaxin-2, CCL25/TECK, CCL26/Eotaxin-3, CCL27/CTACK, CCL28/MEC, CCL3/M1P-1-α, CCL3L1/LD-78-β, CCL4/MIP-1-β, CCL5/RANTES, CCL6/C10, CCL7/MCP-3, CCL8/MCP-2, CCL9/10/MTP-1-γ, CCR, CCR1, CCR10, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CD1, CD10, CD105, CD11a, CD11b, CD11c, CD123, CD13, CD137, CD138, CD14, CD140a, CD146, CD147, CD148, CD15, CD152, CD16, CD164, CD18, CD19, CD2, CD20, CD21, CD22, CD23, CD25, CD26, CD27L, CD28, CD29, CD3, CD30, CD30L, CD32, CD33 (p67 proteins), CD34, CD37, CD38, CD3E, CD4, CD40, CD40L, CD44, CD45, CD46, CD49a, CD49b, CD5, CD51, CD52, CD54, CD55, CD56, CD6, CD61, CD64, CD66e, CD7, CD70, CD74, CD8, CD80 (B7-1), CD89, CD95, CD105, CD158a, CEA, CEACAM5, CFTR, cGMP, CGRP receptor, CINC, CKb8-1, Claudin18, CLC, Clostridium botulinum toxin, Clostridium difficile toxin, Clostridium perfringens toxin, c-Met, CMV, CMV UL, CNTF, CNTN-1, complement factor 3 (C3), complement factor D, corticosteroid-binding globulin, Colony stimulating factor-1 receptor, COX, C-Ret, CRG-2, CRTH2, CT-1, CTACK, CTGF, CTLA-4, CX3CL1/Fractalkine, CX3CR1, CXCL, CXCL1/Gro-α, CXCL10, CXCL11/I-TAC, CXCL12/SDF-l- α/β, CXCL13/BCA-1, CXCL14/BRAK, CXCL15/Lungkine, CXCL16, CXCL16, CXCL2/Gro-β CXCL3/Gro-γ, CXCL3, CXCL4/PF4, CXCL5/ENA-78, CXCL6/GCP-2, CXCL7/NAP-2, CXCL8/IL-8, CXCL9/Mig, CXCLlO/IP-10, CXCR, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, cystatin C, cytokeratin tumor-associated antigen, DAN, DCC, DcR3, DC-SIGN, Decay accelerating factor, Delta-like protein ligand 4, des(1-3)-IGF-1 (brain IGF-1), Dhh, DHICA oxidase, Dickkopf-1, digoxin, Dipeptidyl peptidase IV, DKl, DNAM-1, Dnase, Dpp, DPPIV/CD26, Dtk, ECAD, EDA, EDA-A1, EDA-A2, EDAR, EGF, EGFR (ErbB-1), EGF like domain containing protein 7, Elastase, elastin, EMA, EMMPRIN, ENA, ENA-78, Endosialin, endothelin receptor, endotoxin, Enkephalinase, eNOS, Eot, Eotaxin, Eotaxin-2, eotaxini, EpCAM, Ephrin B2/EphB4, Epha2 tyrosine kinase receptor, epidermal

growth factor receptor (EGFR), ErbB2 receptor, ErbB3 tyrosine kinase receptor, ERCC, erythropoietin (EPO), Erythropoietin receptor, E-selectin, ET-1, Exodus-2, F protein of RSV, F10, F11, F12, F13, F5, F9, Factor Ia, Factor IX, Factor Xa, Factor VII, factor VIII, Factor VIIIc, Fas, FcαR, FcepsilonRI, FcγIIb, FcγRI, FcγRIIa, FcγRIIIa, FcγRIIIb, FcRn, FEN-1, Ferritin, FGF, FGF-19, FGF-2, FGF-2 receptor, FGF-3, FGF-8, FGF-acidic, FGF-basic, FGFR, FGFR-3, Fibrin, fibroblast activation protein (FAP), fibroblast growth factor, fibroblast growth factor-10, fibronectin, FL, FLIP, Flt-3, FLT3 ligand, Folate receptor, follicle stimulating hormone (FSH), Fractalkine (CX3C), free heavy chain, free light chain, FZD1, FZD10, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, G250, Gas 6, GCP-2, GCSF, G-CSF, G-CSF receptor, GD2, GD3, GDF, GDF-1, GDF-15 (MIC-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, GDNF, Gelsolin, GFAP, GF-CSF, GFR-α1, GFR-α2, GFR-α3, GF-β1, gH envelope glycoprotein, GITR, Glucagon, Glucagon receptor, Glucagon-like peptide 1 receptor, Glut 4, Glutamate carboxypeptidase II, glycoprotein hormone receptors, glycoprotein IIb/IIIa (GP IIb/IIIa), Glypican-3, GM-CSF, GM-CSF receptor, gp130, gp140, gp72, granulocyte-CSF (G-CSF), GRO/MGSA, Growth hormone releasing factor, GRO-β, GRO-γ, H. pylori, Hapten (NP-cap or NIP-cap), HB-EGF, HCC, HCC 1, HCMV gB envelope glycoprotein, HCMV UL, Hemopoietic growth factor (HGF), Hep B gp120, heparanase, heparin cofactor II, hepatic growth factor, Bacillus anthracis protective antigen, Hepatitis C virus E2 glycoprotein, Hepatitis E, Hepcidin, Her1, Her2/neu (ErbB-2), Her3 (ErbB-3), Her4 (ErbB-4), herpes simplex virus (HSV) gB glycoprotein, HGF, HGFA, High molecular weight melanoma-associated antigen (HMW-MAA), HIV envelope proteins such as GP120, HIV MIB gp 120 V3 loop, HLA, HLA-DR, HM1.24, HMFG PEM, HMGB-1, HRG, Hrk, HSP47, Hsp90, HSV gD glycoprotein, human cardiac myosin, human cytomegalovirus (HCMV), human growth hormone (hGH), human serum albumin, human tissue-type plasminogen activator (t-PA), Huntingtin, HVEM, IAP, ICAM, ICAM-1, ICAM-3, ICE, ICOS, IFN-α, IFN-β, IFN-γ, IgA, IgA receptor, IgE, IGF, IGF binding proteins, IGF-1, IGF-1 R, IGF-2, IGFBP, IGFR, IL, IL-1, IL-10, IL-10 receptors, IL-11, IL-11 receptors, IL-12, IL-12 receptors, IL-13, IL-13 receptors, IL-15, IL-15 receptors, IL-16, IL-16 receptors, IL-17, IL-17 receptors, IL-18 (IGIF), IL-18 receptors, IL-1α, IL-1β, IL-1 receptors, IL-2, IL-2 receptors, IL-20, IL-20 receptors, IL-21, IL-21 receptors, IL-23, IL-23 receptors, IL-2 receptors, IL-3, IL-3 receptors, IL-31, IL-31 receptors, IL-3 receptors, IL-4, IL-4 receptors IL-5, IL-5 receptors, IL-6, IL-6 receptors, IL-7, IL-7 receptors, IL-8, IL-8 receptors, IL-9, IL-9 receptors, immunoglobulin immune complex, immunoglobulins, INF-α, INF-α receptors, INF-β, INF-β receptors, INF-γ, INF-γ receptors, IFN type-I, IFN type-I receptor, influenza, inhibin, Inhibin α, Inhibin β, iNOS, insulin, Insulin A-chain, Insulin B-chain, Insulin-like growth factor 1, insulin-like growth factor 2, insulin-like growth factor binding proteins, integrin, integrin α2,

integrin α3, integrin α4, integrin α4/β1, integrin α-V/β-3, integrin α-V/β-6, integrin α4/β7, integrin α5/β1, integrin α5/β3, integrin α5/β6, integrin α-δ (αV), integrin α-θ, integrin β1, integrin β2, integrin β3(GPIIb-IIIa), IP-10, I-TAC, JE, kalliklein, Kallikrein 11, Kallikrein 12, Kallikrein 14, Kallikrein 15, Kallikrein 2, Kallikrein 5, Kallikrein 6, Kallikrein L1, Kallikrein L2, Kallikrein L3, Kallikrein L4, kallistatin, KC, KDR, Keratinocyte Growth Factor (KGF), Keratinocyte Growth Factor-2 (KGF-2), KGF, killer immunoglobulin-like receptor, kit ligand (KL), Kit tyrosine kinase, laminin 5, LAMP, LAPP (Amylin, islet-amyloid polypeptide), LAP (TGF- 1), latency associated peptide, Latent TGF-1, Latent TGF-1 bp1, LBP, LDGF, LDL, LDL receptor, LECT2, Lefty, Leptin, leutinizing hormone (LH), Lewis-Y antigen, Lewis-Y related antigen, LFA-1, LFA-3, LFA-3 receptors, Lfo, LIF, LIGHT, lipoproteins, LIX, LKN, Lptn, L-Selectin, LT-a, LT-b, LTB4, LTBP-1, Lung surfactant, Luteinizing hormone, Lymphotactin, Lymphotoxin β Receptor, Lysosphingolipid receptor, Mac-1, macrophage-CSF (M-CSF), MAdCAM, MAG, MAP2, MARC, maspin, MCAM, MCK-2, MCP, MCP-1, MCP-2, MCP-3, MCP-4, MCP-I (MCAF), M-CSF, MDC, MDC (67 a.a.), MDC (69 a.a.), megsin, Mer, MET tyrosine kinase receptor family, METALLOPROTEASES, Membrane glycoprotein OX2, Mesothelin, MGDF receptor, MGMT, MHC (HLA-DR), microbial protein, MIF, MIG, MIP, MIP-1α, MIP-1β, MIP-3α, MIP-3β, MIP-4, 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, monocyte attractant protein, monocyte colony inhibitory factor, mouse gonadotropin-associated peptide, MPIF, Mpo, MSK, MSP, MUC-16, MUC18, mucin (Muc), Muellerian-inhibiting substance, Mug, MuSK, Myelin associated glycoprotein, myeloid progenitor inhibitor factor-1 (MPIF-I), NAIP, Nanobody, NAP, NAP-2, NCA 90, NCAD, N-Cadherin, NCAM, Neprilysin, Neural cell adhesion molecule, neroserpin, Neuronal growth factor (NGF), Neurotrophin-3, Neurotrophin-4, Neurotrophin-6, Neuropilin 1, Neurturin, NGF-β, NGFR, NKG20, N-methionyl human growth hormone, nNOS, NO, Nogo-A, Nogo receptor, non-structural protein type 3 (NS3) from the hepatitis C virus, NOS, Npn, NRG-3, NT, NT-3, NT-4, NTN, OB, OGG1, Oncostatin M, OP-2, OPG, OPN, OSM, OSM receptors, osteoinductive factors, osteopontin, OX40L, OX40R, oxidized LDL, p150, p95, PADPr, parathyroid hormone, PARC, PARP, PBR, PBSF, PCAD, P-Cadherin, PCNA, PCSK9, PDGF, PDGF receptor, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-D, PDK-1, PECAM, PEDF, PEM, PF-4, PGE, PGF, PGI2, PGJ2, PIGF, PIN, PLA2, Placenta growth factor, placental alkaline phosphatase (PLAP), placental lactogen, plasminogen activator inhibitor-1, platelet-growth factor, plgR, PLP, poly glycol chains of different size(e.g. PEG-20, PEG-30, PEG40), PP14, prekallikrein, prion protein, procalcitonin, Programmed cell death protein 1, proinsulin, prolactin, Proprotein convertase PC9, prorelaxin, prostate specific membrane antigen (PSMA), Protein A, Protein C, Protein D, Protein S, Protein Z, PS, PSA, PSCA, PsmAr, PTEN, PTHrp, Ptk, PTN, P-selectin glycoprotein ligand-1, R51,

RAGE, RANK, RANKL, RANTES, relaxin, Relaxin A-chain, Relaxin B-chain, renin, respiratory syncytial virus (RSV) F, Ret, reticulon 4, Rheumatoid factors, RLI P76, RPA2, RPK-1, RSK, RSV Fgp, S100, RON-8, SCF/KL, SCGF, Sclerostin, SDF-1, SDF1 α, SDF1 β, SERINE, Serum Amyloid P, Serum albumin, sFRP-3, Shh, Shiga like toxin II, SIGIRR, SK-1, SLAM, SLPI, SMAC, SMDF, SMOH, SOD, SPARC, sphingosine 1-phosphate receptor 1, Staphylococcal lipoteichoic acid, Stat, STEAP, STEAP-II, stem cell factor (SCF), streptokinase, superoxide dismutase, syndecan-1, TACE, TACI, TAG-72 (tumor-associated glycoprotein-72), TARC, TB, TCA-3, T-cell receptor α/β, TdT, TECK, TEM1, TEM5, TEM7, TEM8, Tenascin, TERT, testicular PLAP-like alkaline phosphatase, TfR, TGF, TGF-α, TGF-β, TGF-β Pan Specific, TGF-β RII, TGF-β RIIb, TGF-β RIII, TGF-β RI (ALK-5), TGF-β1, TGF-β2, TGF-β3, TGF-β4, TGF-β5, TGF-I, Thrombin, thrombopoietin (TPO), Thymic stromal lymphoprotein receptor, Thymus Ck-1, thyroid stimulating hormone (TSH), thyroxine, thyroxine-binding globulin, Tie, TIMP, TIQ, Tissue Factor, tissue factor protease inhibitor, tissue factor protein, TMEFF2, Tmpo, TMPRSS2, TNF receptor I, TNF receptor II, TNF-α, TNF-β, TNF-β2, 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), TNFRSF12A, 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), TNFRSF21 (DR6), TNFRSF22 (DcTRAIL R2 TNFRH2), TNFRSF25 (DR3 Apo-3/LARD/TR-3/TRAMP/WSL-1), 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 (4-1 BB CD137/ILA), TNFRST23 (DcTRAIL R1 TNFRH1), 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 (4-1 BB Ligand CD137 Ligand), TNFα, TNF-β, TNIL-I, toxic metabolite, TP-1, t-PA, Tpo, TRAIL, TRAIL R, TRAIL-R1, TRAIL-R2, TRANCE, transferrin receptor, transforming growth factors (TGF) such as TGF-α and TGF-β, Transmembrane

glycoprotein NMB, Transthyretin, TRF, Trk, TROP-2, Trophoblast glycoprotein, TSG, TSLP, Tumor Necrosis Factor (TNF), tumor-associated antigen CA 125, tumor-associated antigen expressing Lewis Y related carbohydrate, TWEAK, TXB2, Ung, uPAR, uPAR-1, Urokinase, VAP-1, vascular endothelial growth factor (VEGF), vaspin, VCAM, VCAM-1, VECAD, VE-Cadherin, VE-Cadherin-2, VEFGR-1 (flt-1), VEFGR-2, VEGF receptor (VEGFR), VEGFR-3 (flt-4), VEGI, VIM, Viral antigens, VitB12 receptor, Vitronectin receptor, VLA, VLA-1, VLA-4, VNR integrin, von Willebrand Factor (vWF), WIF-1, WNT1, WNT10A, WNT10B, WNT11, WNT16, WNT2, WNT2B/13, WNT3, WNT3A, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT9A, WNT9B, XCL1, XCL2/SCM-1-β, XCL1/Lymphotactin, XCR1, XEDAR, XIAP, XPD, HMGB1, IgA, Aβ, CD81, CD97, CD98, DDR1, DKK1, EREG, Hsp90, IL-17/IL-17R, IL-20/IL-20R, oxidated 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, and S1P and soluble receptor molecules for a hormone or growth factor, which are not anchored to cells in the body fluid of organisms.

"Epitope" means an antigenic determinant in an antigen, and refers to an antigen site to which the antigen-binding domain of an antigen-binding molecule disclosed herein binds. Thus, for example, the epitope can be defined according to its structure. Alternatively, the epitope may be defined according to the antigen-binding activity of an antigen-binding molecule that recognizes the epitope. When the antigen is a peptide or polypeptide, the epitope can be specified by the amino acid residues forming the epitope. Alternatively, when the epitope is a sugar chain, the epitope can be specified by its specific sugar chain structure.

A linear epitope is an epitope that contains an epitope whose primary amino acid sequence is recognized. Such a linear epitope typically contains at least three and most commonly at least five, for example, about 8 to 10 or 6 to 20 amino acids in its specific sequence.

In contrast to the linear epitope, "conformational epitope" is an epitope in which the primary amino acid sequence containing the epitope is not the only determinant of the recognized epitope (for example, the primary amino acid sequence of a conformational epitope is not necessarily recognized by an epitope-defining antibody). Conformational epitopes may contain a greater number of amino acids compared to linear epitopes. A conformational epitope-

recognizing antibody recognizes the three-dimensional structure of a peptide or protein. For example, when a protein molecule folds and forms a three-dimensional structure, amino acids and/or polypeptide main chains that form a conformational epitope become aligned, and the epitope is made recognizable by the antibody. Methods for determining epitope conformations include, for example, X ray crystallography, two-dimensional nuclear magnetic resonance, site-specific spin labeling, and electron paramagnetic resonance, but are not limited thereto. See, for example, Epitope Mapping Protocols in Methods in Molecular Biology (1996), Vol. 66, Morris (ed.).

Binding Activity

Examples of a method for assessing the epitope binding by a test antigen-binding molecule containing an IL-6R antigen-binding domain are described below. According to the examples below, methods for assessing the epitope binding by a test antigen-binding molecule containing an antigen-binding domain for an antigen other than IL-6R, can also be appropriately conducted.

For example, whether a test antigen-binding molecule containing an IL-6R antigen-binding domain recognizes a linear epitope in the IL-6R molecule can be confirmed for example as mentioned below. A linear peptide comprising an amino acid sequence forming the extracellular domain of IL-6R is synthesized for the above purpose. The peptide can be synthesized chemically, or obtained by genetic engineering techniques using a region encoding the amino acid sequence corresponding to the extracellular domain in an IL-6R cDNA. Then, a test antigen-binding molecule containing an IL-6R antigen-binding domain is assessed for its binding activity towards a linear peptide comprising the amino acid sequence forming the extracellular domain. For example, an immobilized linear peptide can be used as an antigen by ELISA to evaluate the binding activity of the antigen-binding molecule towards the peptide. Alternatively, the binding activity towards a linear peptide can be assessed based on the level that the linear peptide inhibits the binding of the antigen-binding molecule to IL-6R-expressing cells. These tests can demonstrate the binding activity of the antigen-binding molecule towards the linear peptide.

Whether a test antigen-binding molecule containing an IL-6R antigen-binding domain recognizes a conformational epitope can be assessed as follows. IL-6R-expressing cells are prepared for the above purpose. A test antigen-binding molecule containing an IL-6R antigen-binding domain can be determined to recognize a conformational epitope when it strongly binds to IL-6R-expressing cells upon contact, but does not substantially bind to an immobilized linear peptide comprising an amino acid sequence forming the extracellular domain of IL-6R. Herein, "not substantially bind" means that the binding activity is 80% or less, generally 50% or less,

preferably 30% or less, and particularly preferably 15% or less compared to the binding activity towards cells expressing human IL-6R.

Methods for assaying the binding activity of a test antigen-binding molecule containing an IL-6R antigen-binding domain towards IL-6R-expressing cells include, for example, the methods described in Antibodies: A Laboratory Manual (Ed Harlow, David Lane, Cold Spring Harbor Laboratory (1988) 359-420). Specifically, the assessment can be performed based on the principle of ELISA or fluorescence activated cell sorting (FACS) using IL-6R-expressing cells as antigen.

In the ELISA format, the binding activity of a test antigen-binding molecule containing an IL-6R antigen-binding domain towards IL-6R-expressing cells can be assessed quantitatively by comparing the levels of signal generated by enzymatic reaction. Specifically, a test antigen-binding molecule is added to an ELISA plate onto which IL-6R-expressing cells are immobilized. Then, the test antigen-binding molecule bound to the cells is detected using an enzyme-labeled antibody that recognizes the test antigen-binding molecule. Alternatively, when FACS is used, a dilution series of a test antigen-binding molecule is prepared, and the antibody binding titer for IL-6R-expressing cells can be determined to compare the binding activity of the test antigen-binding molecule towards IL-6R-expressing cells.

The binding of a test antigen-binding molecule towards an antigen expressed on the surface of cells suspended in buffer or the like can be detected using a flow cytometer. Known flow cytometers include, for example, the following devices:

FACSCanto™ II
FACSAria™
FACSArray™
FACSVantage™ SE
FACSCalibur™ (all are trade names of BD Biosciences)
EPICS ALTRA HyPerSort
Cytomics FC 500
EPICS XL-MCL ADC EPICS XL ADC
Cell Lab Quanta/Cell Lab Quanta SC (all are trade names of Beckman Coulter).

Preferable methods for assaying the binding activity of a test antigen-binding molecule containing an IL-6R antigen-binding domain towards an antigen include, for example, the following method. First, IL-6R-expressing cells are reacted with a test antigen-binding molecule, and then this is stained with an FITC-labeled secondary antibody that recognizes the antigen-binding molecule. The test antigen-binding molecule is appropriately diluted with a suitable buffer to prepare the molecule at a desired concentration. For example, the molecule can be used at a concentration within the range of 10 μg/ml to 10 ng/ml. Then, the fluorescence intensity and

cell count are determined using FACSCalibur (BD). The fluorescence intensity obtained by analysis using the CELL QUEST Software (BD), *i.e.*, the Geometric Mean value, reflects the quantity of antibody bound to cells. That is, the binding activity of a test antigen-binding molecule, which is represented by the quantity of the test antigen-binding molecule bound, can be determined by measuring the Geometric Mean value.

Whether a test antigen-binding molecule containing an IL-6R antigen-binding domain shares a common epitope with another antigen-binding molecule can be assessed based on the competition between the two molecules for the same epitope. The competition between antigen-binding molecules can be detected by cross-blocking assay or the like. For example, the competitive ELISA assay is a preferred cross-blocking assay.

Specifically, in cross-blocking assay, the IL-6R protein immobilized to the wells of a microtiter plate is pre-incubated in the presence or absence of a candidate competitor antigen-binding molecule, and then a test antigen-binding molecule is added thereto. The quantity of test antigen-binding molecule bound to the IL-6R protein in the wells is indirectly correlated with the binding ability of a candidate competitor antigen-binding molecule that competes for the binding to the same epitope. That is, the greater the affinity of the competitor antigen-binding molecule for the same epitope, the lower the binding activity of the test antigen-binding molecule towards the IL-6R protein-coated wells.

The quantity of the test antigen-binding molecule bound to the wells *via* the IL-6R protein can be readily determined by labeling the antigen-binding molecule in advance. For example, a biotin-labeled antigen-binding molecule is measured using an avidin/peroxidase conjugate and appropriate substrate. In particular, cross-blocking assay that uses enzyme labels such as peroxidase is called "competitive ELISA assay". The antigen-binding molecule can also be labeled with other labeling substances that enable detection or measurement. Specifically, radiolabels, fluorescent labels, and such are known.

When the candidate competitor antigen-binding molecule can block the binding by a test antigen-binding molecule containing an IL-6R antigen-binding domain by at least 20%, preferably at least 20 to 50%, and more preferably at least 50% compared to the binding activity in a control experiment conducted in the absence of the competitor antigen-binding molecule, the test antigen-binding molecule is determined to substantially bind to the same epitope bound by the competitor antigen-binding molecule, or compete for the binding to the same epitope.

When the structure of an epitope bound by a test antigen-binding molecule containing an IL-6R antigen-binding domain has already been identified, whether the test and control antigen-binding molecules share a common epitope can be assessed by comparing the binding activities of the two antigen-binding molecules towards a peptide prepared by introducing amino acid mutations into the peptide forming the epitope.

To measure the above binding activities, for example, the binding activities of test and control antigen-binding molecules towards a linear peptide into which a mutation is introduced are compared in the above ELISA format. Besides the ELISA methods, the binding activity towards the mutant peptide bound to a column can be determined by flowing test and control antigen-binding molecules in the column, and then quantifying the antigen-binding molecule eluted in the elution solution. Methods for adsorbing a mutant peptide to a column, for example, in the form of a GST fusion peptide, are known.

Alternatively, when the identified epitope is a conformational epitope, whether test and control antigen-binding molecules share a common epitope can be assessed by the following method. First, IL-6R-expressing cells and cells expressing IL-6R with a mutation introduced into the epitope are prepared. The test and control antigen-binding molecules are added to a cell suspension prepared by suspending these cells in an appropriate buffer such as PBS. Then, the cell suspensions are appropriately washed with a buffer, and an FITC-labeled antibody that recognizes the test and control antigen-binding molecules is added thereto. The fluorescence intensity and number of cells stained with the labeled antibody are determined using FACSCalibur (BD). The test and control antigen-binding molecules are appropriately diluted using a suitable buffer, and used at desired concentrations. For example, they may be used at a concentration within the range of 10 μg/ml to 10 ng/ml. The fluorescence intensity determined by analysis using the CELL QUEST Software (BD), *i.e.*, the Geometric Mean value, reflects the quantity of labeled antibody bound to cells. That is, the binding activities of the test and control antigen-binding molecules, which are represented by the quantity of labeled antibody bound, can be determined by measuring the Geometric Mean value.

In the above method, whether an antigen-binding molecule does "not substantially bind to cells expressing mutant IL-6R" can be assessed, for example, by the following method. First, the test and control antigen-binding molecules bound to cells expressing mutant IL-6R are stained with a labeled antibody. Then, the fluorescence intensity of the cells is determined. When FACSCalibur is used for fluorescence detection by flow cytometry, the determined fluorescence intensity can be analyzed using the CELL QUEST Software. From the Geometric Mean values in the presence and absence of the antigen-binding molecule, the comparison value (ΔGeo-Mean) can be calculated according to the following formula to determine the ratio of increase in fluorescence intensity as a result of the binding by the antigen-binding molecule.

ΔGeo-Mean = Geo-Mean (in the presence of the antigen-binding molecule)
/Geo-Mean (in the absence of the antigen-binding molecule)

The Geometric Mean comparison value (ΔGeo-Mean value for the mutant IL-6R

molecule) determined by the above analysis, which reflects the quantity of a test antigen-binding molecule bound to cells expressing mutant IL-6R, is compared to the ΔGeo-Mean comparison value that reflects the quantity of the test antigen-binding molecule bound to IL-6R-expressing cells. In this case, the concentrations of the test antigen-binding molecule used to determine the ΔGeo-Mean comparison values for IL-6R-expressing cells and cells expressing mutant IL-6R are particularly preferably adjusted to be equal or substantially equal. An antigen-binding molecule that has been confirmed to recognize an epitope in IL-6R is used as a control antigen-binding molecule.

If the ΔGeo-Mean comparison value of a test antigen-binding molecule for cells expressing mutant IL-6R is smaller than the ΔGeo-Mean comparison value of the test antigen-binding molecule for IL-6R-expressing cells by at least 80%, preferably 50%, more preferably 30%, and particularly preferably 15%, then the test antigen-binding molecule "does not substantially bind to cells expressing mutant IL-6R". The formula for determining the Geo-Mean (Geometric Mean) value is described in the CELL QUEST Software User's Guide (BD biosciences). When the comparison shows that the comparison values are substantially equivalent, the epitope for the test and control antigen-binding molecules can be determined to be the same.

Antigen-binding domain

Herein, an "antigen-binding domain" may be of any structure as long as it binds to an antigen of interest. Such domains preferably include, for example:
antibody heavy-chain and light-chain variable regions;
a module of about 35 amino acids called A domain which is contained in the *in vivo* cell membrane protein Avimer (WO 2004/044011, WO 2005/040229);
Adnectin containing the 10Fn3 domain which binds to the protein moiety of fibronectin, a glycoprotein expressed on cell membrane (WO 2002/032925);
Affibody which is composed of a 58-amino acid three-helix bundle based on the scaffold of the IgG-binding domain of Protein A (WO 1995/001937);
Designed Ankyrin Repeat proteins (DARPins) which are a region exposed on the molecular surface of ankyrin repeats (AR) having a structure in which a subunit consisting of a turn comprising 33 amino acid residues, two antiparallel helices, and a loop is repeatedly stacked (WO 2002/020565);
Anticalins and such, which are domains consisting of four loops that support one side of a barrel structure composed of eight circularly arranged antiparallel strands that are highly conserved among lipocalin molecules such as neutrophil gelatinase-associated lipocalin (NGAL) (WO 2003/029462); and

the concave region formed by the parallel-sheet structure inside the horseshoe-shaped structure constituted by stacked repeats of the leucine-rich-repeat (LRR) module of the variable lymphocyte receptor (VLR) which does not have the immunoglobulin structure and is used in the system of acquired immunity in jawless vertebrate such as lampery and hagfish (WO 2008/016854). Preferred antigen-binding domains of the present invention include, for example, those having antibody heavy-chain and light-chain variable regions. Preferred examples of antigen-binding domains include "single chain Fv (scFv)", "single chain antibody", "Fv", "single chain Fv 2 (scFv2)", "Fab", and "F(ab')2".

The antigen-binding domains of antigen-binding molecules of the present invention can bind to an identical epitope. Such epitope can be present, for example, in a protein comprising the amino acid sequence of SEQ ID NO: 15. Alternatively, the epitope can be present in the protein comprising the amino acids at positions 20 to 365 in the amino acid sequence of SEQ ID NO: 15. Alternatively, each of the antigen-binding domains of antigen-binding molecules of the present invention can bind to a different epitope. Herein, the different epitope can be present in, for example, a protein comprising the amino acid sequence of SEQ ID NO: 15. Alternatively, the epitope can be present in the protein comprising the amino acids at positions 20 to 365 in the amino acid sequence of SEQ ID NO: 15.

Calcium-binding motif

The antigen-binding domain of an antigen-binding molecule of the present invention comprises a calcium-binding motif. The calcium-binding motif can be located anywhere within the antigen-binding domain as long as the antigen-binding activity is lower under a low calcium concentration condition than under a high calcium concentration condition. When the antigen-binding domain is an antibody variable region, the calcium-binding motif can be contained in the heavy-chain variable region or light-chain variable region. Alternatively, the calcium-binding motif can be contained in both heavy chains and light chains. In another non-limiting embodiment, the calcium-binding motif can be contained in the framework or CDR sequence of the variable region. Alternatively, the calcium-binding motif can be contained in both framework and CDR sequences.

In a non-limiting embodiment of the present invention, the calcium-binding motif comprises an amino acid residue(s) that alters the antigen-binding activity of the antigen-binding molecule depending on the calcium-ion concentration condition. Such amino acid residues preferably include, for example, amino acids having a metal-chelating activity. Amino acids having a metal-chelating activity preferably include, for example, serine (Ser (S)), threonine (Thr (T)), asparagine (Asn (N)), glutamine (Gln (Q)), aspartic acid (Asp (D)), glutamic acid (Glu (E)), histidine (His (H)), and tyrosine (Tyr (Y)). The calcium-binding motifs in existing antigen-

binding domains that have a lower antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition can be used as a suitable calcium-binding motif of the present invention. As examples of such existing antigen-binding domains, calcium-binding motifs in the variable regions of antibodies that have a lower antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition can be preferably used; but are not limited thereto. Such antibodies include, but are not limited to, for example, IL-6 receptor antibodies comprising SEQ ID NOs: 1 and 2 and IL-6 antibodies comprising SEQ ID NOs: 25 and 26. Furthermore, troponin C, calmodulin, parvalbumin, myosin light chain, and such, which have several calcium ion-binding sites and are assumed to be derived from a common origin in their molecular evolution, are known. Their binding motifs can also be used as a calcium-binding motif of the present invention.

When an antigen-binding domain of the present invention is an antibody variable region, the calcium-binding motif can be contained in its heavy-chain variable region or light-chain variable region. Alternatively, the calcium-binding motif can be contained in both heavy chains and light chains. In another non-limiting embodiment, the calcium-binding motif can be contained in the framework or CDR sequence of the variable region. Alternatively, the calcium-binding motif can be contained in both framework and CDR sequences. The heavy chain or light chain CDR1, CDR2, and/or CDR3 can be designed so that they comprise such calcium-binding motifs. For example, in a non-limiting embodiment of the present invention, the light-chain variable region of an antigen-binding molecule of the present invention can be designed so as to contain the calcium-binding motif of the human antibody light chain variable region of SEQ ID NO: 41, 63, or 64. Such calcium-binding motifs include those in which any one or more of the amino acids at positions 30, 31, 32, 50, and/or 92 according to Kabat's numbering have a metal-chelating activity. In a non-limiting embodiment, such calcium-binding motifs preferably include those in which the same amino acids as one to four amino acids selected from the five amino acids at positions 30, 31, 32, 50, and/or 92 according to Kabat's numbering system in the human antibody light chain variable region of SEQ ID NO: 41, 63, or 64 are contained at the corresponding amino acid positions according to Kabat's numbering system. In this case, it is preferable that amino acids having a metal-chelating activity are contained in the human antibody light chain variable region of SEQ ID NO: 41, 63, or 64 at amino acid positions where amino acids at the corresponding amino acid positions of the five amino acid positions 30, 31, 32, 50, and/or 92 according to Kabat's numbering system in the light chain variable region are not identical to the amino acids at these positions. In another non-limiting embodiment of the present invention, the heavy-chain variable region of an antigen-binding molecule of the present invention can be designed to have, for example, the calcium-binding motif of the heavy-chain variable region of SEQ ID NO: 1. Such calcium-binding motifs include those in which the

amino acids at positions 95, 96, and/or 100a according to Kabat's numbering system have a metal-chelating activity. In another non-limiting embodiment of the present invention, the heavy-chain variable region of an antigen-binding molecule of the present invention can be designed to have, for example, the calcium-binding motif of the heavy-chain variable region of SEQ ID NO: 25. Such calcium-binding motifs include those in which the amino acids at positions 95 and/or 101 according to Kabat's numbering system have a metal-chelating activity. Amino acids having a metal-chelating activity include, for example, serine (Ser (S)), threonine (Thr (T)), asparagine (Asn (N)), glutamine (Gln (Q)), aspartic acid (Asp (D)), glutamic acid (Glu (E)), histidine (His (H)), and tyrosine (Tyr (Y)). Furthermore, the main chain carbonyl groups of amino acids at the positions described above may participate in the calcium ion binding. Surprisingly, as described in the Examples below, calcium ion-binding activity can be conferred to an antigen-binding domain of interest by grafting amino acids from a calcium-binding motif to the antigen-binding domain. It is also possible to appropriately use an EF hand, which is contained in the cadherin domain and calmodulin; C2 domain, which is contained in Protein kinase C; Gla domain, which is contained in blood coagulation protein Factor IX; C-type lectin, which is contained in acyaroglycoprotein receptor and mannose-binding receptor; A domain, which is contained in LDL receptor; Annexin, thrombospondin type-3 domain, and EGF-like domain.

Specificity

"Specific" means that a molecule does not show any significant binding to molecules other than a single or a number of binding partner molecules. Furthermore, "specific" is also used when an antigen-binding domain is specific to a particular epitope among multiple epitopes in an antigen. When an epitope bound by an antigen-binding domain is contained in multiple different antigens, antigen-binding molecules containing the antigen-binding domain can bind to various antigens that have the epitope.

Antibody

Herein, "antibody" refers to a natural immunoglobulin or an immunoglobulin produced by partial or complete synthesis. Antibodies can be isolated from natural sources such as naturally-occurring plasma and serum, or culture supernatants of antibody-producing hybridomas. Alternatively, antibodies can be partially or completely synthesized using techniques such as genetic recombination. Preferred antibodies include, for example, antibodies of an immunoglobulin isotype or subclass belonging thereto. Known human immunoglobulins include antibodies of the following nine classes (isotypes): IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, and IgM. Of these isotypes, antibodies of the present invention include IgG1, IgG2,

IgG3, and IgG4.

Methods for producing an antibody with desired binding activity are known to those skilled in the art. Below is an example that describes a method for producing an antibody that binds to IL-6R (anti-IL-6R antibody). Antibodies that bind to an antigen other than IL-6R can also be produced according to the example described below.

Anti-IL-6R antibodies can be obtained as polyclonal or monoclonal antibodies using known methods. The anti-IL-6R antibodies preferably produced are monoclonal antibodies derived from mammals. Such mammal-derived monoclonal antibodies include antibodies produced by hybridomas or host cells transformed with an expression vector carrying an antibody gene by genetic engineering techniques. "Humanized antibodies" or "chimeric antibodies" are included in the monoclonal antibodies of the present invention.

Monoclonal antibody-producing hybridomas can be produced using known techniques, for example, as described below. Specifically, mammals are immunized by conventional immunization methods using an IL-6R protein as a sensitizing antigen. Resulting immune cells are fused with known parental cells by conventional cell fusion methods. Then, hybridomas producing an anti-IL-6R antibody can be selected by screening for monoclonal antibody-producing cells using conventional screening methods.

Specifically, monoclonal antibodies are prepared as mentioned below. First, the IL-6R gene whose nucleotide sequence is disclosed in SEQ ID NO: 16 can be expressed to produce an IL-6R protein shown in SEQ ID NO: 15, which will be used as a sensitizing antigen for antibody preparation. That is, a gene sequence encoding IL-6R is inserted into a known expression vector, and appropriate host cells are transformed with this vector. The desired human IL-6R protein is purified from the host cells or their culture supernatants by known methods. In order to obtain soluble IL-6R from culture supernatants, for example, a protein consisting of the amino acids at positions 1 to 357 in the IL-6R polypeptide sequence of SEQ ID NO: 15, such as described in Mullberg *et al.* (J. Immunol. (1994) 152 (10), 4958-4968), is expressed as a soluble IL-6R, instead of the IL-6R protein of SEQ ID NO: 15. Purified natural IL-6R protein can also be used as a sensitizing antigen.

The purified IL-6R protein can be used as a sensitizing antigen for immunization of mammals. A partial IL-6R peptide may also be used as a sensitizing antigen. In this case, a partial peptide can be prepared by chemical synthesis based on the amino acid sequence of human IL-6R, or by inserting a partial IL-6R gene into an expression vector for expression. Alternatively, a partial peptide can be produced by degrading an IL-6R protein with a protease. The length and region of the partial IL-6R peptide are not limited to particular embodiments. A preferred region can be arbitrarily selected from the amino acid sequence at amino acid positions 20 to 357 in the amino acid sequence of SEQ ID NO: 15. The number of amino acids forming a

peptide to be used as a sensitizing antigen is preferably at least five or more, six or more, or seven or more. More specifically, a peptide of 8 to 50 residues, more preferably 10 to 30 residues can be used as a sensitizing antigen.

For sensitizing antigen, alternatively it is possible to use a fusion protein prepared by fusing a desired partial polypeptide or peptide of the IL-6R protein with a different polypeptide. For example, antibody Fc fragments and peptide tags are preferably used to produce fusion proteins to be used as sensitizing antigens. Vectors for expression of such fusion proteins can be constructed by fusing in frame genes encoding two or more desired polypeptide fragments and inserting the fusion gene into an expression vector as described above. Methods for producing fusion proteins are described in Molecular Cloning 2nd ed. (Sambrook, J *et al.*, Molecular Cloning 2nd ed., 9.47-9.58 (1989) Cold Spring Harbor Lab. Press). Methods for preparing IL-6R to be used as a sensitizing antigen, and immunization methods using IL-6R are specifically described in WO 2003/000883, WO 2004/022754, WO 2006/006693, and such.

There is no particular limitation on the mammals to be immunized with the sensitizing antigen. However, it is preferable to select the mammals by considering their compatibility with the parent cells to be used for cell fusion. In general, rodents such as mice, rats, and hamsters, rabbits, and monkeys are preferably used.

The above animals are immunized with a sensitizing antigen by known methods. Generally performed immunization methods include, for example, intraperitoneal or subcutaneous administration of a sensitizing antigen into mammals. Specifically, a sensitizing antigen is appropriately diluted with PBS (Phosphate-Buffered Saline), physiological saline, or the like. If desired, a conventional adjuvant such as Freund's complete adjuvant is mixed with the antigen, and the mixture is emulsified. Then, the sensitizing antigen is administered to a mammal several times at 4- to 21-day intervals. Appropriate carriers may be used in immunization with the sensitizing antigen. In particular, when a low-molecular-weight partial peptide is used as the sensitizing antigen, it is sometimes desirable to couple the sensitizing antigen peptide to a carrier protein such as albumin or keyhole limpet hemocyanin for immunization.

Alternatively, hybridomas producing a desired antibody can be prepared using DNA immunization as mentioned below. DNA immunization is an immunization method that confers immunostimulation by expressing a sensitizing antigen in an animal immunized as a result of administering a vector DNA constructed to allow expression of an antigen protein-encoding gene in the animal. As compared to conventional immunization methods in which a protein antigen is administered to animals to be immunized, DNA immunization is expected to be superior in that:

- immunostimulation can be provided while retaining the structure of a membrane protein such as IL-6R; and

- there is no need to purify the antigen for immunization.

In order to prepare a monoclonal antibody of the present invention using DNA immunization, first, a DNA expressing an IL-6R protein is administered to an animal to be immunized. The IL-6R-encoding DNA can be synthesized by known methods such as PCR. The obtained DNA is inserted into an appropriate expression vector, and then this is administered to an animal to be immunized. Preferably used expression vectors include, for example, commercially-available expression vectors such as pcDNA3.1. Vectors can be administered to an organism using conventional methods. For example, DNA immunization is performed by using a gene gun to introduce expression vector-coated gold particles into cells in the body of an animal to be immunized. Antibodies that recognized IL-6R can also be produced by the methods described in WO 2003/104453.

After immunizing a mammal as described above, an increase in the titer of an IL-6R-binding antibody is confirmed in the serum. Then, immune cells are collected from the mammal, and then subjected to cell fusion. In particular, splenocytes are preferably used as immune cells.

A mammalian myeloma cell is used as a cell to be fused with the above-mentioned immune cells. The myeloma cells preferably comprise a suitable selection marker for screening. A selection marker confers characteristics to cells for their survival (or death) under a specific culture condition. Hypoxanthine-guanine phosphoribosyltransferase deficiency (hereinafter abbreviated as HGPRT deficiency) and thymidine kinase deficiency (hereinafter abbreviated as TK deficiency) are known as selection markers. Cells with HGPRT or TK deficiency have hypoxanthine-aminopterin-thymidine sensitivity (hereinafter abbreviated as HAT sensitivity). HAT-sensitive cells cannot synthesize DNA in a HAT selection medium, and are thus killed. However, when the cells are fused with normal cells, they can continue DNA synthesis using the salvage pathway of the normal cells, and therefore they can grow even in the HAT selection medium.

HGPRT-deficient and TK-deficient cells can be selected in a medium containing 6-thioguanine, 8-azaguanine (hereinafter abbreviated as 8AG), or 5'-bromodeoxyuridine, respectively. Normal cells are killed because they incorporate these pyrimidine analogs into their DNA. Meanwhile, cells that are deficient in these enzymes can survive in the selection medium, since they cannot incorporate these pyrimidine analogs. In addition, a selection marker referred to as G418 resistance provided by the neomycin-resistant gene confers resistance to 2-deoxystreptamine antibiotics (gentamycin analogs). Various types of myeloma cells that are suitable for cell fusion are known.

For example, myeloma cells including the following cells can be preferably used:
P3(P3x63Ag8.653) (J. Immunol. (1979) 123 (4), 1548-1550);
P3x63Ag8U.1 (Current Topics in Microbiology and Immunology (1978)81, 1-7);

NS-1 (C. Eur. J. Immunol. (1976)6 (7), 511-519);
MPC-11 (Cell (1976) 8 (3), 405-415);
SP2/0 (Nature (1978) 276 (5685), 269-270);
FO (J. Immunol. Methods (1980) 35 (1-2), 1-21);
S194/5.XX0.BU.1 (J. Exp. Med. (1978) 148 (1), 313-323);
R210 (Nature (1979) 277 (5692), 131-133), etc.

Cell fusions between the immunocytes and myeloma cells are essentially carried out using known methods, for example, a method by Kohler and Milstein *et al.* (Methods Enzymol. (1981) 73: 3-46).

More specifically, cell fusion can be carried out, for example, in a conventional culture medium in the presence of a cell fusion-promoting agent. The fusion-promoting agents include, for example, polyethylene glycol (PEG) and Sendai virus (HVJ). If required, an auxiliary substance such as dimethyl sulfoxide is also added to improve fusion efficiency.

The ratio of immune cells to myeloma cells may be determined at one's own discretion, preferably, for example, one myeloma cell for every one to ten immunocytes. Culture media to be used for cell fusions include, for example, media that are suitable for the growth of myeloma cell lines, such as RPMI1640 medium and MEM medium, and other conventional culture medium used for this type of cell culture. In addition, serum supplements such as fetal calf serum (FCS) may be preferably added to the culture medium.

For cell fusion, predetermined amounts of the above immune cells and myeloma cells are mixed well in the above culture medium. Then, a PEG solution (for example, the average molecular weight is about 1,000 to 6,000) prewarmed to about 37°C is added thereto at a concentration of generally 30% to 60% (w/v). This is gently mixed to produce desired fusion cells (hybridomas). Then, an appropriate culture medium mentioned above is gradually added to the cells, and this is repeatedly centrifuged to remove the supernatant. Thus, cell fusion agents and such which are unfavorable to hybridoma growth can be removed.

The hybridomas thus obtained can be selected by culture using a conventional selective medium, for example, HAT medium (a culture medium containing hypoxanthine, aminopterin, and thymidine). Cells other than the desired hybridomas (non-fused cells) can be killed by continuing culture in the above HAT medium for a sufficient period of time. Typically, the period is several days to several weeks. Then, hybridomas producing the desired antibody are screened and singly cloned by conventional limiting dilution methods.

The hybridomas thus obtained can be selected using a selection medium based on the selection marker possessed by the myeloma used for cell fusion. For example, HGPRT- or TK-deficient cells can be selected by culture using the HAT medium (a culture medium containing hypoxanthine, aminopterin, and thymidine). Specifically, when HAT-sensitive myeloma cells

are used for cell fusion, cells successfully fused with normal cells can selectively proliferate in the HAT medium. Cells other than the desired hybridomas (non-fused cells) can be killed by continuing culture in the above HAT medium for a sufficient period of time. Specifically, desired hybridomas can be selected by culture for generally several days to several weeks. Then, hybridomas producing the desired antibody are screened and singly cloned by conventional limiting dilution methods.

Desired antibodies can be preferably selected and singly cloned by screening methods based on known antigen/antibody reaction. For example, an IL-6R-binding monoclonal antibody can bind to IL-6R expressed on the cell surface. Such a monoclonal antibody can be screened by fluorescence activated cell sorting (FACS). FACS is a system that assesses the binding of an antibody to cell surface by analyzing cells contacted with a fluorescent antibody using laser beam, and measuring the fluorescence emitted from individual cells.

To screen for hybridomas that produce a monoclonal antibody of the present invention by FACS, IL-6R-expressing cells are first prepared. Cells preferably used for screening are mammalian cells in which IL-6R is forcedly expressed. As control, the activity of an antibody to bind to cell-surface IL-6R can be selectively detected using non-transformed mammalian cells as host cells. Specifically, hybridomas producing an anti-IL-6R monoclonal antibody can be isolated by selecting hybridomas that produce an antibody which binds to cells forced to express IL-6R, but not to host cells.

Alternatively, the activity of an antibody to bind to immobilized IL-6R-expressing cells can be assessed based on the principle of ELISA. For example, IL-6R-expressing cells are immobilized to the wells of an ELISA plate. Culture supernatants of hybridomas are contacted with the immobilized cells in the wells, and antibodies that bind to the immobilized cells are detected. When the monoclonal antibodies are derived from mouse, antibodies bound to the cells can be detected using an anti-mouse immunoglobulin antibody. Hybridomas producing a desired antibody having the antigen-binding ability are selected by the above screening, and they can be cloned by a limiting dilution method or the like.

Monoclonal antibody-producing hybridomas thus prepared can be passaged in a conventional culture medium, and stored in liquid nitrogen for a long period.

The above hybridomas are cultured by a conventional method, and desired monoclonal antibodies can be prepared from the culture supernatants. Alternatively, the hybridomas are administered to and grown in compatible mammals, and monoclonal antibodies are prepared from the ascites. The former method is suitable for preparing antibodies with high purity.

Antibodies encoded by antibody genes that are cloned from antibody-producing cells such as the above hybridomas can also be preferably used. A cloned antibody gene is inserted into an appropriate vector, and this is introduced into a host to express the antibody encoded by

the gene. Methods for isolating antibody genes, inserting the genes into vectors, and transforming host cells have already been established, for example, by Vandamme *et al.* (Eur. J. Biochem. (1990) 192(3), 767-775). Methods for producing recombinant antibodies are also known as described below.

For example, a cDNA encoding the variable region (V region) of an anti-IL-6R antibody is prepared from hybridoma cells expressing the anti-IL-6R antibody. For this purpose, total RNA is first extracted from hybridomas. Methods used for extracting mRNAs from cells include, for example:

- the guanidine ultracentrifugation method (Biochemistry (1979) 18(24), 5294-5299), and
- the AGPC method (Anal. Biochem. (1987) 162(1), 156-159)

Extracted mRNAs can be purified using the mRNA Purification Kit (GE Healthcare Bioscience) or such. Alternatively, kits for extracting total mRNA directly from cells, such as the QuickPrep mRNA Purification Kit (GE Healthcare Bioscience), are also commercially available. mRNAs can be prepared from hybridomas using such kits. cDNAs encoding the antibody V region can be synthesized from the prepared mRNAs using a reverse transcriptase. cDNAs can be synthesized using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Co.) or such. Furthermore, the SMART RACE cDNA amplification kit (Clontech) and the PCR-based 5'-RACE method (Proc. Natl. Acad. Sci. USA (1988) 85(23), 8998-9002; Nucleic Acids Res. (1989) 17(8), 2919-2932) can be appropriately used to synthesize and amplify cDNAs. In such a cDNA synthesis process, appropriate restriction enzyme sites described below may be introduced into both ends of a cDNA.

The cDNA fragment of interest is purified from the resulting PCR product, and then this is ligated to a vector DNA. A recombinant vector is thus constructed, and introduced into *E. coli* or such. After colony selection, the desired recombinant vector can be prepared from the colony-forming *E. coli*. Then, whether the recombinant vector has the cDNA nucleotide sequence of interest is tested by a known method such as the dideoxy nucleotide chain termination method.

The 5'-RACE method which uses primers to amplify the variable region gene is conveniently used for isolating the gene encoding the variable region. First, a 5'-RACE cDNA library is constructed by cDNA synthesis using RNAs extracted from hybridoma cells as a template. A commercially available kit such as the SMART RACE cDNA amplification kit is appropriately used to synthesize the 5'-RACE cDNA library.

The antibody gene is amplified by PCR using the prepared 5'-RACE cDNA library as a template. Primers for amplifying the mouse antibody gene can be designed based on known antibody gene sequences. The nucleotide sequences of the primers vary depending on the immunoglobulin subclass. Therefore, it is preferable that the subclass is determined in advance using a commercially available kit such as the Iso Strip mouse monoclonal antibody isotyping kit

(Roche Diagnostics).

Specifically, for example, primers that allow amplification of genes encoding γ1, γ2a, γ2b, and γ3 heavy chains and κ and λ light chains are used to isolate mouse IgG-encoding genes. In general, a primer that anneals to a constant region site close to the variable region is used as a 3'-side primer to amplify an IgG variable region gene. Meanwhile, a primer attached to a 5' RACE cDNA library construction kit is used as a 5'-side primer.

PCR products thus amplified are used to reshape immunoglobulins composed of a combination of heavy and light chains. A desired antibody can be selected using the IL-6R-binding activity of a reshaped immunoglobulin as an indicator. For example, when the objective is to isolate an antibody against IL-6R, it is more preferred that the binding of the antibody to IL-6R is specific. An IL-6R-binding antibody can be screened, for example, by the following steps:

(1) contacting an IL-6R-expressing cell with an antibody comprising the V region encoded by a cDNA isolated from a hybridoma;

(2) detecting the binding of the antibody to the IL-6R-expressing cell; and

(3) selecting an antibody that binds to the IL-6R-expressing cell.

Methods for detecting the binding of an antibody to IL-6R-expressing cells are known. Specifically, the binding of an antibody to IL-6R-expressing cells can be detected by the above-described techniques such as FACS. Immobilized samples of IL-6R-expressing cells are appropriately used to assess the binding activity of an antibody.

Preferred antibody screening methods that use the binding activity as an indicator also include panning methods using phage vectors. Screening methods using phage vectors are advantageous when the antibody genes are isolated from heavy-chain and light-chain subclass libraries from a polyclonal antibody-expressing cell population. Genes encoding the heavy-chain and light-chain variable regions can be linked by an appropriate linker sequence to form a single-chain Fv (scFv). Phages presenting scFv on their surface can be produced by inserting a gene encoding scFv into a phage vector. The phages are contacted with an antigen of interest. Then, a DNA encoding scFv having the binding activity of interest can be isolated by collecting phages bound to the antigen. This process can be repeated as necessary to enrich scFv having the binding activity of interest.

After isolation of the cDNA encoding the V region of the anti-IL-6R antibody of interest, the cDNA is digested with restriction enzymes that recognize the restriction sites introduced into both ends of the cDNA. Preferred restriction enzymes recognize and cleave a nucleotide sequence that occurs in the nucleotide sequence of the antibody gene at a low frequency. Furthermore, a restriction site for an enzyme that produces a sticky end is preferably introduced into a vector to insert a single-copy digested fragment in the correct orientation. The cDNA encoding the V region of the anti-IL-6R antibody is digested as described above, and this

is inserted into an appropriate expression vector to construct an antibody expression vector. In this case, if a gene encoding the antibody constant region (C region) and a gene encoding the above V region are fused in-frame, a chimeric antibody is obtained. Herein, "chimeric antibody" means that the origin of the constant region is different from that of the variable region. Thus, in addition to mouse/human heterochimeric antibodies, human/human allochimeric antibodies are included in the chimeric antibodies of the present invention. A chimeric antibody expression vector can be constructed by inserting the above V region gene into an expression vector that already has the constant region. Specifically, for example, a recognition sequence for a restriction enzyme that excises the above V region gene can be appropriately placed on the 5' side of an expression vector carrying a DNA encoding a desired antibody constant region (C region). A chimeric antibody expression vector is constructed by fusing in frame the two genes digested with the same combination of restriction enzymes.

To produce an anti-IL-6R monoclonal antibody, antibody genes are inserted into an expression vector so that the genes are expressed under the control of an expression regulatory region. The expression regulatory region for antibody expression includes, for example, enhancers and promoters. Furthermore, an appropriate signal sequence may be attached to the amino terminus so that the expressed antibody is secreted to the outside of cells. For example, a peptide having the amino acid sequence MGWSCIILFLVATATGVHS (SEQ ID NO: 113) can be used as a signal sequence. Meanwhile, other appropriate signal sequences may be attached. The expressed polypeptide is cleaved at the carboxyl terminus of the above sequence, and the resulting polypeptide is secreted to the outside of cells as a mature polypeptide. Then, appropriate host cells are transformed with the expression vector, and recombinant cells expressing the anti-IL-6R antibody-encoding DNA are obtained.

DNAs encoding the antibody heavy chain (H chain) and light chain (L chain) are separately inserted into different expression vectors to express the antibody gene. An antibody molecule having the H and L chains can be expressed by co-transfecting the same host cell with vectors into which the H-chain and L-chain genes are respectively inserted. Alternatively, host cells can be transformed with a single expression vector into which DNAs encoding the H and L chains are inserted (see WO 1994011523).

There are various known host cell/expression vector combinations for antibody preparation by introducing isolated antibody genes into appropriate hosts. All of these expression systems are applicable to isolation of the antigen-binding domains of the present invention. Appropriate eukaryotic cells used as host cells include animal cells, plant cells, and fungal cells. Specifically, the animal cells include, for example, the following cells.

(1) mammalian cells: CHO, COS, myeloma, baby hamster kidney (BHK), HeLa, Vero, or such;
(2) amphibian cells: Xenopus oocytes, or such; and

(3) insect cells: sf9, sf21, Tn5, or such.

In addition, as a plant cell, an antibody gene expression system using cells derived from the *Nicotiana* genus such as *Nicotiana tabacum* is known. Callus cultured cells can be appropriately used to transform plant cells.

Furthermore, the following cells can be used as fungal cells:

- yeasts: the *Saccharomyces* genus such as *Saccharomyces cerevisiae*, and the *Pichia* genus such as *Pichia pastoris*; and
- filamentous fungi: the *Aspergillus* genus such as *Aspergillus niger*.

Furthermore, antibody gene expression systems that utilize prokaryotic cells are also known. For example, when using bacterial cells, *E. coli* cells, *Bacillus subtilis* cells, and such can suitably be utilized in the present invention. Expression vectors carrying the antibody genes of interest are introduced into these cells by transfection. The transfected cells are cultured *in vitro*, and the desired antibody can be prepared from the culture of transformed cells.

In addition to the above-described host cells, transgenic animals can also be used to produce a recombinant antibody. That is, the antibody can be obtained from an animal into which the gene encoding the antibody of interest is introduced. For example, the antibody gene can be constructed as a fusion gene by inserting in frame into a gene that encodes a protein produced specifically in milk. Goat β-casein or such can be used, for example, as the protein secreted in milk. DNA fragments containing the fused gene inserted with the antibody gene is injected into a goat embryo, and then this embryo is introduced into a female goat. Desired antibodies can be obtained as a protein fused with the milk protein from milk produced by the transgenic goat born from the embryo-recipient goat (or progeny thereof). In addition, to increase the volume of milk containing the desired antibody produced by the transgenic goat, hormones can be administered to the transgenic goat as necessary (Ebert, K. M. *et al.*, Bio/Technology (1994) 12 (7), 699-702).

When an antigen-binding molecule described herein is administered to human, an antigen-binding domain derived from a genetically recombinant antibody that has been artificially modified to reduce the heterologous antigenicity against human and such, can be appropriately used as the antigen-binding domain of the complex. Such genetically recombinant antibodies include, for example, humanized antibodies. These modified antibodies are appropriately produced by known methods.

An antibody variable region used to produce the antigen-binding domain of an antigen-binding molecule described herein is generally formed by three complementarity-determining regions (CDRs) that are separated by four framework regions (FRs). CDR is a region that substantially determines the binding specificity of an antibody. The amino acid sequences of CDRs are highly diverse. On the other hand, the FR-forming amino acid sequences often have

high identity even among antibodies with different binding specificities. Therefore, generally, the binding specificity of a certain antibody can be introduced to another antibody by CDR grafting.

A humanized antibody is also called a reshaped human antibody. Specifically, humanized antibodies prepared by grafting the CDR of a non-human animal antibody such as a mouse antibody to a human antibody and such are known. Common genetic engineering techniques for obtaining humanized antibodies are also known. Specifically, for example, overlap extension PCR is known as a method for grafting a mouse antibody CDR to a human FR. In overlap extension PCR, a nucleotide sequence encoding a mouse antibody CDR to be grafted is added to primers for synthesizing a human antibody FR. Primers are prepared for each of the four FRs. It is generally considered that when grafting a mouse CDR to a human FR, selecting a human FR that has high identity to a mouse FR is advantageous for maintaining the CDR function. That is, it is generally preferable to use a human FR comprising an amino acid sequence which has high identity to the amino acid sequence of the FR adjacent to the mouse CDR to be grafted.

Nucleotide sequences to be ligated are designed so that they will be connected to each other in frame. Human FRs are individually synthesized using the respective primers. As a result, products in which the mouse CDR-encoding DNA is attached to the individual FR-encoding DNAs are obtained. Nucleotide sequences encoding the mouse CDR of each product are designed so that they overlap with each other. Then, complementary strand synthesis reaction is conducted to anneal the overlapping CDR regions of the products synthesized using a human antibody gene as template. Human FRs are ligated *via* the mouse CDR sequences by this reaction.

The full length V region gene, in which three CDRs and four FRs are ultimately ligated, is amplified using primers that anneal to its 5'- or 3'-end, which are added with suitable restriction enzyme recognition sequences. An expression vector for humanized antibody can be produced by inserting the DNA obtained as described above and a DNA that encodes a human antibody C region into an expression vector so that they will ligate in frame. After the recombinant vector is transfected into a host to establish recombinant cells, the recombinant cells are cultured, and the DNA encoding the humanized antibody is expressed to produce the humanized antibody in the cell culture (see, European Patent Publication No. EP 239400 and International Patent Publication No. WO 1996/002576).

By qualitatively or quantitatively measuring and evaluating the antigen-binding activity of the humanized antibody produced as described above, one can suitably select human antibody FRs that allow CDRs to form a favorable antigen-binding site when ligated through the CDRs. Amino acid residues in FRs may be substituted as necessary, so that the CDRs of a reshaped

human antibody form an appropriate antigen-binding site. For example, amino acid sequence mutations can be introduced into FRs by applying the PCR method used for grafting a mouse CDR into a human FR. More specifically, partial nucleotide sequence mutations can be introduced into primers that anneal to the FR. Nucleotide sequence mutations are introduced into the FRs synthesized by using such primers. Mutant FR sequences having the desired characteristics can be selected by measuring and evaluating the activity of the amino acid-substituted mutant antibody to bind to the antigen by the above-mentioned method (Cancer Res. (1993) 53: 851-856).

Alternatively, desired human antibodies can be obtained by immunizing transgenic animals having the entire repertoire of human antibody genes (see WO 1993/012227; WO 1992/003918; WO 1994/002602; WO 1994/025585; WO 1996/034096; WO 1996/033735) by DNA immunization.

Furthermore, techniques for preparing human antibodies by panning using human antibody libraries are also known. For example, the V region of a human antibody is expressed as a single-chain antibody (scFv) on phage surface by the phage display method. Phages expressing an scFv that binds to the antigen can be selected. The DNA sequence encoding the human antibody V region that binds to the antigen can be determined by analyzing the genes of selected phages. The DNA sequence of the scFv that binds to the antigen is determined. An expression vector is prepared by fusing the V region sequence in frame with the C region sequence of a desired human antibody, and inserting this into an appropriate expression vector. The expression vector is introduced into cells appropriate for expression such as those described above. The human antibody can be produced by expressing the human antibody-encoding gene in the cells. These methods are already known (see WO 1992/001047; WO 1992/020791; WO 1993/006213; WO 1993/011236; WO 1993/019172; WO 1995/001438; WO 1995/015388).

In addition to the techniques described above, techniques of B cell cloning (identification of each antibody-encoding sequence, cloning and its isolation; use in constructing expression vector in order to prepare each antibody (IgG1, IgG2, IgG3, or IgG4 in particular); and such) such as described in Bernasconi *et al.* (Science (2002) 298: 2199-2202) or in WO 2008/081008 can be appropriately used to isolate antibody genes.

EU numbering system

According to the methods used in the present invention, amino acid positions assigned to antibody CDR and FR are specified according to Kabat's numbering (Sequences of Proteins of Immunological Interest (National Institute of Health, Bethesda, Md., 1987 and 1991)). Herein, when an antigen-binding molecule is an antibody or antigen-binding fragment, variable region amino acids are indicated according to Kabat's numbering system, while constant region amino

acids are indicated according to EU numbering system based on Kabat's amino acid positions.

Antigen uptake into cells or promoting the antigen uptake into cells

Herein, "antigen uptake into cells" mediated by antigen-binding molecules means that antigens are incorporated into cells *via* endocytosis. Herein, "promoting the antigen uptake into cells" means increasing the rate of cellular uptake of an antigen-binding molecule that has bound to an antigen in plasma and/or decreasing the amount of antigen recycled to plasma after uptake. In the present invention, the rate of uptake into cells may be enhanced compared to that of the antigen-binding molecule before reducing its antigen-binding activity under a low calcium concentration condition to be lower than that under a high calcium concentration condition. Thus, in the present invention, whether antigen uptake into cells is facilitated by an antigen-binding molecule can be assessed based on an increase in the rate of antigen uptake into cells. The rate of antigen uptake into cells can be calculated, for example, by monitoring over time reduction in the antigen concentration in the culture medium containing human FcRn-expressing cells after adding the antigen and antigen-binding molecule to the medium, or monitoring over time the amount of antigen uptake into human FcRn-expressing cells.

Using methods of the present invention for facilitating the rate of antigen-binding molecule-mediated antigen uptake into cells, for example, the rate of antigen elimination from the plasma can be enhanced by administering antigen-binding molecules. Thus, whether antigen-binding molecule-mediated antigen uptake into cells is facilitated can also be assessed, for example, by testing whether the rate of antigen elimination from the plasma is accelerated or whether the plasma antigen concentration is reduced by administering an antigen-binding molecule. Specifically, the reduction of the antigen concentration in plasma can also be promoted by administering antigen-binding molecules of the present invention.

The number of times of antigen binding by one antigen-binding molecule

Herein, "the number of times of antigen binding by one antigen-binding molecule" means the number of times of antigen binding that can be achieved by one antigen-binding molecule until it is eliminated due to degradation. Herein, "increasing the number of times of antigen binding by one antigen-binding molecule" means increasing the number of cycles that can be achieved by one antigen-binding molecule until it is eliminated due to degradation when defining as "one cycle" the process in which the antigen-binding molecule binds to an antigen in plasma, and the antigen-binding molecule bound to the antigen is taken up into cells, and dissociates from the antigen in an endosome, and then the antigen-binding molecule returns to plasma. In the present invention, the number of cycles may be increased compared to that of an antigen-binding molecule whose antigen-binding activity under a low calcium concentration

condition is not lower than that under a high calcium concentration condition, or that of the antigen-binding molecule before reducing its antigen-binding activity under a low calcium concentration condition to be lower than that under a high calcium concentration condition. Thus, whether the number of cycles is increased can be assessed by testing whether "the uptake into cells is promoted" as described above or whether "the plasma retention is improved" as described below.

Improvement of plasma retention

Herein, "improvement of the plasma retention" is interchangeable with "enhancement of the pharmacokinetics", "improvement of the pharmacokinetics", "superior pharmacokinetics", "enhancement of the plasma retention", "excellence in plasma retention", or "prolongation of the plasma retention". These phrases are synonymous.

Herein, "improvement of plasma retention" means not only prolongation of the period until elimination from the plasma (for example, until the antigen-binding molecule is degraded intracellularly or the like and cannot return to the plasma) after administration of the antigen-binding molecule to animals such as humans, mice, rats, monkeys, rabbits, and dogs, but also prolongation of the plasma retention of the antigen-binding molecule in a form that allows antigen binding (for example, in an antigen-free form of the antigen-binding molecule) during the period of administration to elimination due to degradation. Specifically, "improvement of plasma retention" also includes prolongation of the period until elimination due to degradation of the antigen-binding molecule not bound to antigens (the antigen-free form of antigen-binding molecule).

The antigen-binding molecule in plasma cannot bind to a new antigen if the antigen-binding molecule has already bound to an antigen. Thus, the longer the period that the antigen-binding molecule is not bound to an antigen, the longer the period that it can bind to a new antigen (the higher the chance of binding to another antigen). This enables reduction of the time period that an antigen is free of the antigen-binding molecule *in vivo* and prolongation of the period that an antigen is bound to the antigen-binding molecule. The plasma concentration of the antigen-free form of antigen-binding molecule can be increased and the period that the antigen is bound to the antigen-binding molecule can be prolonged by accelerating the antigen elimination from the plasma by administration of the antigen-binding molecule. Specifically, as used herein, "improvement of the plasma retention of an antigen-binding molecule" includes improvement of any pharmacokinetic parameter (such as prolongation of half-life in plasma, prolongation of mean plasma retention time, or reduction of clearance in plasma) of an antigen-free antigen-binding molecule of the present invention, prolongation of the period where an antigen is bound to the antigen-binding molecule after administration of the antigen-binding

molecule, and enhancement of antigen elimination from plasma by the antigen-binding molecule, as compared to an antigen-free antigen-binding molecule whose antigen-binding activity under a low calcium concentration condition is not lower than that under a high calcium concentration condition, or an antigen-free antigen-binding molecule before reducing its antigen-binding activity under a low calcium concentration condition to be lower than that under a high calcium concentration condition.

Whether the pharmacokinetics parameters are improved can be assessed by determining any one of the parameters, half-life in plasma, mean plasma retention time, and plasma clearance for the antigen-binding molecule or the antigen-free form thereof ("Pharmacokinetics: Enshu-niyoru Rikai (Understanding through practice)" Nanzando). For example, the plasma concentration of the antigen-binding molecule or antigen-free form thereof is determined after administration of the antigen-binding molecule to mice, rats, monkeys, rabbits, dogs, or humans. Then, each parameter is determined. When the plasma half-life or mean plasma retention time is prolonged, the plasma retention of the antigen-binding molecule can be judged to be improved. The parameters can be determined by methods known to those skilled in the art. The parameters can be appropriately assessed, for example, by noncompartmental analysis using the pharmacokinetics analysis software WinNonlin (Pharsight) according to the appended instruction manual. The plasma concentration of antigen-free form of antigen-binding molecule can be determined by methods known to those skilled in the art, for example, using the assay method described in Clin Pharmacol. 2008 Apr; 48(4): 406-17.

Herein, "improvement of the plasma retention" also includes prolongation of the period where an antigen is bound to an antigen-binding molecule after administration of the antigen-binding molecule. Whether the period where an antigen is bound to an antigen-binding molecule after administration of the antigen-binding molecule is prolonged can be assessed based on the time until the increase in the concentration (ratio) by measuring the plasma concentration of the antigen-binding molecule-unbound antigen (free antigen), or the ratio of the concentration of the antigen-binding molecule-unbound antigen (concentration of the free antigen) to the total antigen concentration.

In the present invention, the "antigen concentration in plasma" can be determined by measuring the plasma concentration of an antigen-binding molecule-free antigen, or the ratio of the concentration of the antigen-binding molecule-free antigen to the total antigen concentration, using methods known to those skilled in the art, for example, measurement methods described in Pharm Res. 2006 Jan; 23(1): 95-103.

Alternatively, when an antigen exhibits a particular function *in vivo*, whether the antigen is bound to an antigen-binding molecule that neutralizes the antigen function (antagonistic molecule) can be assessed by testing whether the antigen function is neutralized. Whether the

antigen function is neutralized can be assessed by assaying an *in vivo* marker that reflects the antigen function. Whether the antigen is bound to an antigen-binding molecule that activates the antigen function (agonistic molecule) can be assessed by assaying an *in vivo* marker that reflects the antigen function.

Determination of the plasma concentration of antigen-binding molecule-free antigen and ratio of the concentration of antigen-binding molecule-free antigen to the total antigen concentration, *in vivo* marker assay, and such measurements are not particularly limited; however, the assays are preferably carried out after a certain period of time has passed after administration of the antigen-binding molecule. In the present invention, the period after administration of the antigen-binding molecule is not particularly limited; those skilled in the art can determine the appropriate period depending on the properties and the like of the administered antigen-binding molecule. Such periods include, for example, one day after administration of the antigen-binding molecule, three days after administration of the antigen-binding molecule, seven days after administration of the antigen-binding molecule, 14 days after administration of the antigen-binding molecule, and 28 days after administration of the antigen-binding molecule.

In the present invention, improvement of plasma retention in human is preferred. When the plasma retention in human is difficult to determine, it may be predicted based on the plasma retention in mice (for example, normal mice, human antigen-expressing transgenic mice, human FcRn-expressing transgenic mice) or monkeys (for example, cynomolgus monkeys).

Dissociation of an antigen within a cell from an extracellularly-bound antigen-binding molecule

The present invention is also applicable as a method for promoting the dissociation of an antigen within a cell from an extracellularly-bound antigen-binding molecule. In the present invention, the antigen may dissociate from the antigen-binding molecule anywhere in a cell; however, it is preferred that the antigen dissociates within an early endosome. In the present invention, "an antigen dissociates within a cell from an extracellularly-bound antigen-binding molecule" does not necessarily mean that every antigen which has been taken up into a cell by extracellularly binding to the antigen-binding molecule dissociates from the antigen-binding molecule within the cell. It is acceptable that the proportion of the antigen that dissociates from the antigen-binding molecule within a cell is higher compared to an antigen-binding molecule whose antigen-binding activity under a low calcium concentration condition is not lower than that under a high calcium concentration condition, or the antigen-binding molecule before reducing the antigen-binding activity under a low calcium concentration condition to be lower than that under a high calcium concentration condition. The method for promoting the dissociation of an antigen within a cell from an extracellularly-bound antigen-binding molecule

can also be referred to as a method for conferring to an antigen-binding molecule a property that facilitates promotion of the intracellular uptake of the antigen-binding molecule bound to an antigen, and promotion of the intracellular dissociation of the antigen from the antigen-binding molecule.

Extracellular release in an antigen-free form of an antigen-binding molecule that has been taken up into a cell in an antigen-bound form

The present invention is also applicable as a method for enhancing the extracellular release in an antigen-free form of an antigen-binding molecule that has been taken up into a cell in an antigen-bound form. In the present invention, "extracellular release in an antigen-free form of an antigen-binding molecule that has been taken up into a cell in an antigen-bound form" does not necessarily mean that every antigen-binding molecule that has been bound to an antigen and taken up into a cell is released in an antigen-free form to the outside of a cell. It is acceptable that the proportion of the antigen-binding molecule that is released in an antigen-free form to the outside of cells is higher compared to an antigen-binding molecule whose antigen-binding activity under a low calcium concentration condition is not lower than that under a high calcium concentration condition, or the antigen-binding molecule before reducing its antigen-binding activity under a low calcium concentration condition to be lower than that under a high calcium concentration condition. It is preferred that the antigen-binding molecule released to the outside of a cell retains the antigen-binding activity. The method for promoting the extracellular release in an antigen-free form of an antigen-binding molecule that has been taken up into a cell in an antigen-bound form can also be referred to as a method for conferring to an antigen-binding molecule a property that facilitates promotion of the intracellular uptake of the antigen-binding molecule bound to an antigen, and promotion of the extracellular release of the antigen-binding molecule in an antigen-free form.

Calcium concentration condition

Herein, the low calcium concentration condition typically means the concentration of ionized calcium is 0.1 μM to 30 μM, preferably 0.5 μM to 10 μM, and particularly preferably 1 μM to 5 μM, which is comparable to the concentration of ionized calcium in the early endosome *in vivo*. Meanwhile, herein, the high calcium concentration condition typically means that the concentration of ionized calcium is 100 μM to 10 mM, preferably 200 μM to 5 mM, and particularly preferably 0.5 mM to 2.5 mM, which is comparable to the concentration of ionized calcium in plasma (blood) *in vivo*.

Thus, herein, "the antigen-binding activity of an antigen-binding molecule is lower under a low calcium concentration condition than under a high calcium concentration condition"

means that the antigen-binding activity of an antigen-binding molecule is lower at an ionized calcium concentration of 0.1 μM to 30 μM than at an ionized calcium concentration of 100 μM to 10 mM. It preferably means that the antigen-binding activity of an antigen-binding molecule is lower at an ionized calcium concentration of 0.5 μM to 10 μM than at an ionized calcium concentration of 200 μM to 5 mM. Particularly preferably, it means that the antigen-binding activity is lower at the concentration of ionized calcium in the early endosome *in vivo* than at the concentration of ionized calcium in plasma *in vivo*; specifically, it means that the antigen-binding activity of an antigen-binding molecule is lower at an ionized calcium concentration of 1 μM to 5 μM than at an ionized calcium concentration of 0.5 mM to 2.5 mM.

Meanwhile, as used herein, the phrase "the antigen-binding activity of an antigen-binding molecule is lower under a low calcium concentration condition than under a high calcium concentration condition" is interchangeable with the phrase "the antigen-binding activity of an antigen-binding molecule is higher under a high calcium concentration condition than under a low calcium concentration condition". The phrase "the antigen-binding activity of an antigen-binding molecule is lower under a low calcium concentration condition than under a high calcium concentration condition" also means that the antigen-binding activity of an antigen-binding molecule under a low calcium concentration condition is reduced to be lower than that under a high calcium concentration condition or the antigen-binding activity of an antigen-binding molecule under a high calcium concentration condition is increased to be higher than that under a low calcium concentration condition, by modifying an amino acid sequence in the antigen-binding molecule, etc. That is, in the present invention, the ratio between the antigen-binding activity of an antigen-binding molecule under a low calcium concentration condition and that under a high calcium concentration condition may be increased. For example, in an embodiment, the ratio of KD (Ca 3 μM)/KD (Ca 2 mM) may be increased as described below. The ratio between the antigen-binding activity of an antigen-binding molecule under a low calcium concentration condition and that under a high calcium concentration condition may be increased, for example, by lowering the antigen-binding activity under a low calcium concentration condition through selection of an antigen-binding molecule with low antigen-binding activity under a low calcium concentration condition, or through modification of an amino acid sequence in the antigen-binding molecule; or by increasing the antigen-binding activity under a high calcium concentration condition through selection of an antigen-binding molecule with high antigen-binding activity under a high calcium concentration condition, or through modification of an amino acid sequence in the antigen-binding molecule; or by both of them.

Herein, the expression "the antigen-binding ability is weaker under a low calcium concentration condition than under a high calcium concentration condition", is sometimes used

instead of the phrase "the antigen-binding activity is lower under a low calcium concentration condition than under a high calcium concentration condition". Furthermore, the expression, "weakening the antigen-binding ability under a low calcium concentration condition to be lower than that under a high calcium concentration condition", is sometimes used instead of the phrase "reducing the antigen-binding activity under a low calcium concentration condition to be lower than that under a high calcium concentration condition".

FcRn

Unlike Fcγ receptor belonging to the immunoglobulin superfamily, FcRn, particularly human FcRn, is structurally similar to polypeptides of major histocompatibility complex (MHC) class I, exhibiting 22% to 29% sequence identity to class I MHC molecules (Ghetie *el al.*, Immunol. Today (1997) 18 (12): 592-598). FcRn is expressed as a heterodimer consisting of soluble β or light chain (β2 microglobulin) complexed with transmembrane α or heavy chain. Like MHC, FcRn α chain comprises three extracellular domains (α1, α2, and α3) and its short cytoplasmic domain anchors the protein onto the cell surface. α1 and α2 domains interact with the FcRn-binding domain of the antibody Fc region (Raghavan *et al.*, Immunity (1994) 1: 303-315).

FcRn is expressed in maternal placenta and york sac of mammals, and is involved in mother-to-fetus IgG transfer. In addition, in neonatal small intestine of rodents, where FcRn is expressed, FcRn is involved in transfer of maternal IgG across brush border epithelium from ingested colostrum or milk. FcRn is expressed in a variety of other tissues and endothelial cell systems of various species. FcRn is also expressed in adult human endothelia, muscular blood vessels, and hepatic sinusoidal capillaries. FcRn is believed to play a role in maintaining the plasma IgG concentration by mediating recycling of IgG to serum upon binding to IgG. Typically, binding of FcRn to IgG molecules is strictly pH dependent. The optimal binding is observed in an acidic pH range below 7.0.

Human FcRn whose precursor is a polypeptide having the signal sequence of SEQ ID NO: 17 (the polypeptide with the signal sequence is shown in SEQ ID NO: 18) forms a complex with human β2-microglobulin *in vivo*. Soluble human FcRn complexed with β2-microglobulin is produced by using conventional recombinant expression techniques. FcRn-binding domains of the present invention can be assessed for their binding activity to such a soluble human FcRn complexed with β2-microglobulin. Herein, unless otherwise specified, human FcRn refers to a form capable of binding to an FcRn-binding domain of the present invention. Examples include a complex between human FcRn and human β2-microglobulin.

FcRn-binding domain

The antigen-binding molecules of the present invention have an antigen-binding domain and a human FcRn-binding domain. The human FcRn-binding domain is not particularly limited, as long as the antigen-binding molecules exhibit the human FcRn-binding activity at acidic pH and/or neutral pH. Alternatively, the domain may have a direct or indirect human FcRn-binding activity. Such domains include, for example, the Fc region of IgG-type immunoglobulin, albumin, albumin domain 3, anti-human FcRn antibodies, anti-human FcRn peptides, and anti-human FcRn scaffold molecules, all of which have the activity to directly bind to human FcRn; and molecules that bind to IgG or albumin, which have the activity to indirectly bind to human FcRn. Such preferred domains of the present invention have human FcRn-binding activity in the acidic and neutral pH ranges. It is possible to use the domains without any alteration as long as they already have human FcRn-binding activity in the acidic and neutral pH ranges. When the domains have only weak or no human FcRn-binding activity in the acidic and/or neutral pH ranges, the human FcRn-binding activity may be conferred by altering amino acids in the antigen-binding molecules. However, it is preferred that human FcRn-binding activity in the acidic and/or neutral pH ranges is conferred by altering amino acids in the human FcRn-binding domain. Alternatively, amino acids in the domains that already have human FcRn-binding activity in the acidic and/or neutral pH ranges may be altered to increase the human FcRn-binding activity. Desired amino acid alterations in the human FcRn-binding domain can be selected by comparing the human FcRn-binding activity in the acidic and/or neutral pH ranges before and after amino acid alteration.

The preferred human FcRn-binding domain is a region that directly binds to human FcRn. Such preferred human FcRn-binding regions include, for example, antibody Fc regions. Meanwhile, regions capable of binding to a polypeptide such as albumin or IgG, which has human FcRn-binding activity, can indirectly bind to human FcRn *via* albumin, IgG, or such. Thus, such a human FcRn-binding region of the present invention may be a region that binds to a polypeptide having an activity of binding to albumin or IgG. In particular, a human-FcRn-binding domain with a greater human FcRn-binding activity at neutral pH is preferred. A human-FcRn-binding domain with a greater human FcRn-binding activity at neutral pH may be selected in advance. Alternatively, the human FcRn-binding activity at neutral pH may be conferred or increased by modifying an amino acid in an antigen-binding molecule.

Appropriate conditions, other than the pH at which the human FcRn-binding activity is determined, can be selected by those skilled in the art. The conditions are not particularly limited. For example, the measurements can be conducted at 37°C using MES buffer, as described in WO 2009/125825. Meanwhile, the human FcRn-binding activity of an antigen-binding molecule can be determined by methods known to those skilled in the art, for example, by using Biacore (GE Healthcare) or the like. The activity of binding between an antigen-

binding molecule and human FcRn can be assessed by loading human FcRn or the antigen-binding molecule as an analyte to a chip onto which the antigen-binding molecule or human FcRn is immobilized, respectively.

Herein, the human FcRn-binding activity at acidic pH means the human FcRn-binding activity at pH 4.0 to 6.5, preferably the human FcRn-binding activity at pH 5.5 to 6.5, and particularly preferably the human FcRn-binding activity at pH 5.8 to 6.0, which is comparable to pH in the early endosome *in vivo*. Meanwhile, the human FcRn-binding activity at neutral pH means the human FcRn-binding activity at pH 6.7 to 10.0, preferably the human FcRn-binding activity at pH 7.0 to pH 8.0, and particularly preferably the human FcRn-binding activity at pH 7.4, which is comparable to pH in plasma *in vivo*.

The human FcRn-binding activity at neutral pH can be conferred to or increased in an antigen-binding molecule by modifying an amino acid in the molecule. For example, when the Fc region of an IgG-type immunoglobulin is used as the human-FcRn-binding domain, the human FcRn-binding activity at neutral pH can be conferred to or increased in an antigen-binding molecule by modifying an amino acid in the human-FcRn-binding domain. Preferred Fc region of IgG-type immunoglobulin to be altered includes, for example, the Fc region of a human natural IgG (IgG1, IgG2, IgG3, or IgG4). Amino acids at any sites may be altered to other amino acids as long as the human FcRn-binding activity is conferred or increased at neutral pH. When the antigen-binding molecule has a human IgG1 Fc region as the human FcRn-binding domain, it is preferred that the molecule has alterations that potentiate the binding to human FcRn at neutral pH as compared to that of the human natural IgG1. Amino acids where such alteration can be achieved include, for example, amino acids at positions 221 to 225, 227, 228, 230, 232, 233 to 241, 243 to 252, 254 to 260, 262 to 272, 274, 276, 278 to 289, 291 to 312, 315 to 320, 324, 325, 327 to 339, 341, 343, 345, 360, 362, 370, 375 to 378, 380, 382, 385 to 387, 389, 396, 414, 416, 423, 424, 426 to 438, 440, and 442 (EU numbering). More specifically, such amino acid alterations include, for example, those listed in Table 1. The human FcRn binding of the Fc region of an IgG-type immunoglobulin at neutral pH can be enhanced (potentiated) by using the alterations described above. Furthermore, alterations that can potentiate the binding to human FcRn in the acidic pH range as compared to the human natural IgG1 are shown as an example in Table 2. When appropriate alterations that can also potentiate the binding to human FcRn at neutral pH range are selected from the above-described alterations, they are applicable to the present invention.

"Alteration of amino acids" or "amino acid alteration" of an FcRn-binding domain comprises alteration of an amino acid sequence in a parent FcRn-binding domain to a different amino acid sequences. Any FcRn-binding domain can be used as a parent FcRn-binding domain, as long as variants prepared by modifying the parent FcRn-binding domain can bind to human

FcRn in the neutral pH range. Furthermore, an FcRn-binding domain modified from a parent FcRn-binding domain which has been already modified can also be used preferably as an FcRn-binding domain of the present invention. The "parent FcRn-binding domain" can refer to the polypeptide itself, a composition comprising the parent FcRn-binding domain, or a polynucleotide sequence encoding the parent FcRn-binding domain. Parent FcRn-binding domains can comprise a known Fc region produced *via* recombination described briefly in section "Antibodies". The origin of parent FcRn-binding domains is not limited, and they may be obtained from human or any nonhuman organisms. Such organisms preferably include mice, rats, guinea pigs, hamsters, gerbils, cats, rabbits, dogs, goats, sheep, bovines, horses, camels and organisms selected from nonhuman primates. In another embodiment, parent FcRn-binding domains can also be obtained from cynomolgus monkeys, marmosets, rhesus monkeys, chimpanzees, or humans. Parent FcRn-binding domains can be obtained preferably from human IgG1; however, they are not limited to any particular IgG class. This means that an Fc region of human IgG1, IgG2, IgG3, or IgG4 can be used appropriately as a parent FcRn-binding domain, and herein also means that an Fc region of an arbitrary IgG class or subclass derived from any organisms described above can be preferably used as a parent FcRn-binding domain. Examples of naturally-occurring IgG mutants or modified forms are described in published documents (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; WO 2009/086320; WO 2008/092117; WO 2007/041635; and WO 2006/105338); however, they are not limited to the examples.

Examples of alterations include those with one or more mutations, for example, mutations by substitution of different amino acid residues for amino acids of parent FcRn-binding domains, by insertion of one or more amino acid residues into parent FcRn-binding domains, or by deletion of one or more amino acids from parent FcRn-binding domains. Preferably, the amino acid sequences of altered FcRn-binding domains comprise at least a part of the amino acid sequence of a non-natural FcRn-binding domain. Such variants necessarily have sequence identity or similarity less than 100% to their parent FcRn-binding domain. In a preferred embodiment, the variants have amino acid sequence identity or similarity about 75% to less than 100%, more preferably about 80% to less than 100%, even more preferably about 85% to less than 100%, still more preferably about 90% to less than 100%, and yet more preferably about 95% to less than 100% to the amino acid sequence of their parent FcRn-binding domain. In a non-limiting embodiment of the present invention, at least one amino acid is different between a modified FcRn-binding domain of the present invention and its parent FcRn-binding domain. Amino acid difference between a modified FcRn-binding domain of the present invention and its parent FcRn-binding domain can also be preferably specified based on amino acid differences at above-described particular amino acid positions according to EU numbering

system.

Furthermore, alterations that can potentiate the binding to human FcRn in the acidic pH range as compared to the parent human IgG are shown as an example in Table 2. When appropriate alterations that can also potentiate the binding to human FcRn in the neutral pH range are selected from the above-described alterations, they are applicable to the present invention. Meanwhile, combinations of alterations that can potentiate the binding of Fv4-IgG1 to human FcRn under acidic conditions are shown in Tables 6-1 and 6-2. Particularly preferred amino acids to be altered in the parent human IgG Fc region include, for example, amino acids at positions 237, 238, 239, 248, 250, 252, 254, 255, 256, 257, 258, 265, 270, 286, 289, 297, 298, 303, 305, 307, 308, 309, 311, 312, 314, 315, 317, 325, 332, 334, 360, 376, 380, 382, 384, 385, 386, 387, 389, 424, 428, 433, 434, and 436 (EU numbering).

Particularly preferred alterations include, for example,

an amino acid substitution of Met for Gly at position 237;

an amino acid substitution of Ala for Pro at position 238;

an amino acid substitution of Lys for Ser at position 239;

an amino acid substitution of Ile for Lys at position 248;

an amino acid substitution of Ala, Phe, Ile, Met, Gln, Ser, Val, Trp, or Tyr for Thr at position 250;

an amino acid substitution of Phe, Trp, or Tyr for Met at position 252;

an amino acid substitution of Thr for Ser at position 254;

an amino acid substitution of Glu for Arg at position 255;

an amino acid substitution of Asp, Glu, or Gln for Thr at position 256;

an amino acid substitution of Ala, Gly, Ile, Leu, Met, Asn, Ser, Thr, or Val for Pro at position 257;

an amino acid substitution of His for Glu at position 258;

an amino acid substitution of Ala for Asp at position 265;

an amino acid substitution of Phe for Asp at position 270;

an amino acid substitution of Ala, or Glu for Asn at position 286;

an amino acid substitution of His for Thr at position 289;

an amino acid substitution of Ala for Asn at position 297;

an amino acid substitution of Gly for Ser at position 298;

an amino acid substitution of Ala for Val at position 303;

an amino acid substitution of Ala for Val at position 305;

an amino acid substitution of Ala, Asp, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Val, Trp, or Tyr for Thr at position 307;

an amino acid substitution of Ala, Phe, Ile, Leu, Met, Pro, Gln, or Thr for Val at position 308;

an amino acid substitution of Ala, Asp, Glu, Pro, or Arg for Leu or Val at position 309;
an amino acid substitution of Ala, His, or Ile for Gln at position 311;
an amino acid substitution of Ala, or His for Asp at position 312;
an amino acid substitution of Lys, or Arg for Leu at position 314;
an amino acid substitution of Ala, or His for Asn at position 315;
an amino acid substitution of Ala for Lys at position 317;
an amino acid substitution of Gly for Asn at position 325;
an amino acid substitution of Val for Ile at position 332;
an amino acid substitution of Leu for Lys at position 334;
an amino acid substitution of His for Lys at position 360;
an amino acid substitution of Ala for Asp at position 376;
an amino acid substitution of Ala for Glu at position 380;
an amino acid substitution of Ala for Glu at position 382;
an amino acid substitution of Ala for Asn or Ser at position 384;
an amino acid substitution of Asp, or His for Gly at position 385;
an amino acid substitution of Pro for Gln at position 386;
an amino acid substitution of Glu for Pro at position 387;
an amino acid substitution of Ala, or Ser for Asn at position 389;
an amino acid substitution of Ala for Ser at position 424;
an amino acid substitution of Ala, Asp, Phe, Gly, His, Ile, Lys, Leu, Asn, Pro, Gln, Ser, Thr, Val, Trp, or Tyr for Met at position 428;
an amino acid substitution of Lys for His at position 433;
an amino acid substitution of Ala, Phe, His, Ser, Trp, or Tyr for Asn at position 434;
and an amino acid substitution of His for Tyr or Phe at position 436 (EU numbering) in the parent IgG Fc region.

Meanwhile, the number of amino acids to be altered is not particularly limited; and it is possible to alter amino acids at only a single site or at two or more sites. Combinations of two or more amino acid alterations include, for example, those shown in Table 3. Meanwhile, combinations of alterations that can potentiate the binding to human FcRn in the acidic pH range as compared to the parent human IgG are shown in Tables 4-1 to 4-5. When appropriate combinations of alterations that can also potentiate the binding to human FcRn in the neutral pH range are selected from the above-described alterations, they are applicable to the present invention. Furthermore, combinations of alterations that can potentiate the binding of Fv4-IgG1 to human FcRn under neutral conditions are shown in Tables 5-1 and 5-2.

The human FcRn-binding activity of an antigen-binding molecule in the neutral pH range can be increased by substituting at least one amino acid selected from these amino acids

with a different amino acid.

[Table 1]

POSITION	AMINO ACID ALTERATION
256	P
280	K
339	T
385	H
428	L
434	W, Y, F, A, H

[Table 2]

POSITION	AMINO ACID ALTERATION
221	Y, K
222	Y
223	E, K
224	Y, E
225	E, K, W
227	K, E, G
228	Y, K, G
230	E, G
232	K
233	R, S, M, T, W, Y, G
234	H, R, E, I, V, F, D, Y, G
235	Y, V, N, S, T, Q, D
236	I, V, K, P, E, Q, H, W, Y, D, T, M, A, F, S, N, R
237	I, W, S, T, E, R, N, Q, K, H, D, P, L, M
238	A, L, D, S, T, H, W, V, I, G, M, F, E, K
239	M, R, T, G, V, E, D, L, A
240	I, M, T
241	E, W, L
243	E, W
244	L
245	R
246	Y, H
247	D
248	Y
249	P, Q, Y, H
250	I, E, Q
251	T, D
252	Y, W, Q
254	H
255	E, Y, H
256	A
257	A, I, M, N, S, V, T, L, Y, C
258	D, Y, H, A
259	I, F, N
260	S, D, E, H, Y
262	L, E
263	I
264	F, A, I, T, M, S, D
265	R, P, G, A
266	I
267	K, E, A
268	E, M
269	M, W, K, P, I, S, G, V, F, Y, A
270	K, S, I, A
271	A, V, S, Y, I, T
272	A, L, R, I, D, H, V, W, Y, P, T
274	M, F, G, E, I, T, N
276	D, F, H, R, L, V, W, A
278	R, S, V, M, N, I, L, D
279	A, D, G, H, M, N, Q, R, S, T, W, Y, C, I
281	D, Y
282	G, K, E, Y
283	A, D, F, G, H, I, K, L, N, P, Q, R, S, T, W, Y
284	T, L, Q, E
285	N, Y, W, Q, K, E, D, Y
286	F, L, Y, E, P, D, K, A
287	S, H
288	N, P, Y, H, D, I, V, C, E, G, L, Q, R
289	H
291	Q, H
292	Y, E, D
293	V
294	I, K, G
295	V, T
296	E, I, L
298	F, E, T, H

POSITION	AMINO ACID ALTERATION
299	W, F, H, Y
300	K, A, G, V, M, Q, N, E
301	E
302	I
303	Y, E, A
304	N, T
305	A, H
306	Y
307	A, E, M, G, Q, H
308	A, R, F, C, Y, W, N, H
311	A, I, K, L, M, V, W, T, H
312	A, P, H
315	T, H
316	K
317	A, P, H
318	N, T, R, L, Y
319	L, I, W, H, M, V, A
320	L, W, H, N
324	T, D
325	F, M, D
326	A
327	D, K, M, Y, H, L
328	G, A, W, R, F
329	K, R, W
330	G, W, V, P, H, F
331	L, F, Y
332	F, H, K, L, M, R, S, W, T, Q, E, Y, D, N, V
333	L, F, M, A
334	A
335	H, F, N, V, M, W, I, S, P, L
336	E, K
337	A
338	A
339	N, W
341	P
343	E, H, K, Q, R, T, Y
360	H, A
362	A
375	R
376	A, G, I, M, P, T, V
377	K
378	Q, D, N, W
380	A, N, S, T, Q, R, H
382	A, F, H, I, K, L, M, N, Q, R, S, T, V, W, Y
385	N, E
386	H
387	H, Q
414	A
423	N
424	A
426	H, L, V, R
427	N
428	F
429	Q
430	A, F, G, H, I, K, L, M, N, Q, R, S, T, V, Y
431	H, K
432	H
433	P
434	Q, T, M, S,
435	K
436	I, L, T
437	H
438	K, L, T, W
440	K
442	K

[Table 3]

COMBINATION OF AMINO ACID ALTERATION
M252Y/S254T/T256E
M252Y/S254T/T256E/H433K/N434F/Y436H
H433K/N434F/Y436H
T307A/E380A
T307A/E380A/N434H
T307A/E380A/N434A
N434H/N315H
N434H/T289H
N434H/T370A/E380A
T250Q/M428L
T250Q/N434A
M252W/N434A
M252Y/N434A
T256A/N434A
T256D/N434A
T256E/N434A
T256S/N434A
P257I/Q311I
T307A/N434A
T307E/N434A
T307Q/N434A
V308P/N434A
L309G/N434A
Q311H/N434A
Q311R/N434A
N315D/N434A
A378V/N434A
E380S/N434A
E382V/N434A
S424E/N434A
M428L/N434A
N434A/Y436I
T437Q/N434A
T437R/N434A

[Table 4-1]

COMBINATION OF AMINO ACID ALTERATION
L234I/L235D
G236A/V308F/I332E
G236R/L328R
G236A/I332E/N434S
S239E/V264I/A330Y/I332E
S239E/V264I/I332E
S239E/V264I/S298A/A330Y/I332E
S239D/D265H/N297D/I332E
S239D/E272Y/I332E
S239D/E272S/I332E
S239D/E272I/I332E
S239D/N297D/I332E
S239D/K326T/I332E
S239Q/I332Q
S239Q/I332N
S239D/I332D
S239D/I332E
S239Q/I332E
S239E/I332E
F241W/F243W
F241Y/F243Y/V262T/V264T
F241W/F243W/V262A/V264A
F241L/V262I
F243L/V262I/V264W
F243L/K288D/R292P/Y300L/V305I/P396L/H435K
F243L/K288D/R292P/Y300L/H435K
F243L/R292P/Y300L/V305I/P396L/H435K
P245G/V308F
T250I/V259I/V308F
T250I/V308F
T250I/V308F/N434S
T250Q/V308F/M428L
T250Q/M428L
L251L/N434S
L251N/N434S
L251F/N434S
L251V/N434S
L251M/N434S
T252L/T254S/T256F
M252Y/S254T/T256E/N434M
M252Y/S254T/T256E/M428L/N434S
M252Y/S254T/T256E
M252Y/S254T/T256E/V308F
M252Y/S254T/T256E/N434S
M252Y/S254T/T256E/N434A
M252Y/S254T/T256E/M428L
M252Y/S254T/T256E/T307Q
M252F/T256D
M252Y/T256Q
M252Y/P257L
M252Y/P257N
M252Y/V259I
M252Y/V279Q
M252Y/V308P/N434Y
M252Q/V308F
M252Y/V308F

Table 4-2 is a continuation of Table 4-1.

[Table 4-2]

M252Q/V308F/N434S
M252Y/V308F/M428L
M252Y/V308F/N434M
M252Y/V308F/N434S
M252Y/Y319I
M252Q/M428L/N434S
M252Y/M428L
M252Y/N434M
M252Y/N434S
M252Y/N434A
M252Y/N434Y
S254T/V308F
R255H/N434A
R255Q/N434S
R255H/N434S
T256V/V308F
T256P/Q311I
T256P/I332E
T256P/I332F/S440Y
T256P/E430Q
T256P/N434H
T256E/N434Y
T256P/S440Y
P257Y/V279Q
P257L/V279E
P257N/V279Q
P257N/V279E
P257N/V279Y
P257L/V279Q
P257N/ 281S
P257L/ 281S
P257N/V284E
P257N/L306Y
P257L/V306Y
P257L/V308F
P257N/V308Y
P257I/Q311I/N434H
P257L/Q311V
P257L/G385N
P257L/M428L
P257I/E430Q
P257I/N434H
P257L/N434Y
E258H/N434A
E258H/N434H
V259I/T307Q/V308F
V259I/V308F
V259I/V308F/Y319L
V259I/V308F/Y319I
V259A/V308F
V259I/V308F/N434M
V259I/V308F/N434S
V259I/V308F/M428L/N434S
V259I/V308F/M428L
V259I/Y319I
V259I/Y319I/N434S
V259I/M428L
V259I/M428L/N434S
V259I/N434S

Table 4-3 is a continuation of Table 4-2.

[Table 4-3]

V259I/N434Y
V264I/A330L/I332E
V264I/I332E
D265F/N297E/I332E
S267L/A327S
E272R/V279L
V279E/V284E
V279Q/L306Y
V279Y/V308F
V279Q/V308F
V279Q/G385H
^281S/V308Y
^281S/V308F
^281S/N434Y
E283F/V284E
V284E/V308F
V284E/G385H
K288A/N434A
K288D/H435K
K288V/H435D
T289H/N434A
T289H/N434H
L306I/V308F
T307P/V308F
T307Q/V308F/N434S
T307Q/V308F/Y319L
T307S/V308F
T307Q/V308F
T307A/E310A/N434A
T307Q/E380A/N434A
T307Q/M428L
T307Q/N434M
T307I/N434S
T307V/N434S
T307Q/N434S
T307Q/N434Y
V308T/L309P/Q311S
V308F/L309Y
V308F/Q311V
V308F/Y319F
V308F/Y319I/N434M
V308F/Y319I
V308F/Y319L
V308F/Y319I/M428L
V308F/Y319I/M428L/N434S
V308F/Y319L/N434S
V308F/I332E
V308F/G385H
V308F/M428L/N434M
V308F/M428L
V308F/M428L/N434S
V308P/N434Y
V308F/N434M
V308F/N434S
V308F/N434Y
Q311G/N434S
Q311D/N434S
Q311E/N434S
Q311N/N434S

Table 4-4 is a continuation of Table 4-3.

[Table 4-4]

Q311Y/N434S
Q311F/N434S
Q311W/N434S
Q311A/N434S
Q311K/N434S
Q311T/N434S
Q311R/N434S
Q311L/N434S
Q311M/N434S
Q311V/N434S
Q311I/N434S
Q311A/N434Y
D312H/N434A
D312H/N434H
L314Q/N434S
L314V/N434S
L314M/N434S
L314F/N434S
L314I/N434S
N315H/N434A
N315H/N434H
Y319I/V308F
Y319I/M428L
Y319I/M428L/N434S
Y319I/N434M
Y319I/N434S
L328H/I332E
L328N/I332E
L328E/I332E
L328I/I332E
L328Q/I332E
L328D/I332E
L328R/M428L/N434S
A330L/I332E
A330Y/I332E
I332E/D376V
I332E/N434S
P343R/E345D
D376V/E430Q
D376V/E430R
D376V/N434H
E380A/N434A
G385R/Q386T/P387R/N389P
G385D/Q386P/N389S
N414F/Y416H
M428L/N434M
M428L/N434S
M428L/N434A
M428L/N434Y
I1429N/N434S
E430D/N434S
E430T/N434S
E430S/N434S
E430A/N434S
E430F/N434S
E430Q/N434S
E430L/N434S
E430I/N434S
A431T/N434S

Table 4-5 is a continuation of Table 4-4.

[Table 4-5]

A431S/N434S
A431G/N434S
A431V/N434S
A431N/N434S
A431F/N434S
A431H/N434S
L432F/N434S
L432N/N434S
L432Q/N434S
L432H/N434S
L432G/N434S
L432I/N434S
L432V/N434S
L432A/N434S
H433K/N434F
H433L/N434S
H433M/N434S
H433A/N434S
H433V/N434S
H433K/N434S
H433S/N434S
H433P/N434S
N434S/M428L
N434S/Y436D
N434S/Y436Q
N434S/Y436M
N434S/Y436G
N434S/Y436E
N434S/Y436F
N434S/Y436T
N434S/Y436R
N434S/Y436S
N434S/Y436H
N434S/Y436K
N434S/Y436L
N434S/Y436V
N434S/Y436W
N434S/Y436I
N434S/T437I

[Table 5-1]

VARIANT NAME	KD (M)	AMINO ACID ALTERATION
IgG1	ND	NONE
IgG1-v1	3.2E-06	M252Y/S254T/T256E
IgG1-v2	8.1E-07	N434W
IgG1-F3	2.5E-06	N434Y
IgG1-F4	5.8E-06	N434S
IgG1-F5	6.8E-06	N434A
IgG1-F7	5.6E-06	M252Y
IgG1-F8	4.2E-06	M252W
IgG1-F9	1.4E-07	M252Y/S254T/T256E/N434Y
IgG1-F10	6.9E-08	M252Y/S254T/T256E/N434W
IgG1-F11	3.1E-07	M252Y/N434Y
IgG1-F12	1.7E-07	M252Y/N434W
IgG1-F13	3.2E-07	M252W/N434Y
IgG1-F14	1.8E-07	M252W/N434W
IgG1-F19	4.6E-07	P257L/N434Y
IgG1-F20	4.6E-07	V308F/N434Y
IgG1-F21	3.0E-08	M252Y/V308P/N434Y
IgG1-F22	2.0E-06	M428L/N434S
IgG1-F25	9.2E-09	M252Y/S254T/T256E/V308P/N434W
IgG1-F26	1.0E-06	I332V
IgG1-F27	7.4E-06	G237M
IgG1-F29	1.4E-06	I332V/N434Y
IgG1-F31	2.8E-06	G237M/V308F
IgG1-F32	8.0E-07	S254T/N434W
IgG1-F33	2.3E-06	S254T/N434Y
IgG1-F34	2.8E-07	T256E/N434W
IgG1-F35	8.4E-07	T256E/N434Y
IgG1-F36	3.6E-07	S254T/T256E/N434W
IgG1-F37	1.1E-06	S254T/T256E/N434Y
IgG1-F38	1.0E-07	M252Y/S254T/N434W
IgG1-F39	3.0E-07	M252Y/S254T/N434Y
IgG1-F40	8.2E-08	M252Y/T256E/N434W
IgG1-F41	1.5E-07	M252Y/T256E/N434Y
IgG1-F42	1.0E-06	M252Y/S254T/T256E/N434A
IgG1-F43	1.7E-06	M252Y/N434A
IgG1-F44	1.1E-06	M252W/N434A
IgG1-F47	2.4E-07	M252Y/T256Q/N434W
IgG1-F48	3.2E-07	M252Y/T256Q/N434Y
IgG1-F49	5.1E-07	M252F/T256D/N434W
IgG1-F50	1.2E-06	M252F/T256D/N434Y
IgG1-F51	8.1E-06	N434F/Y436H
IgG1-F52	3.1E-06	H433K/N434F/Y436H
IgG1-F53	1.0E-06	I332V/N434W
IgG1-F54	8.4E-08	V308P/N434W
IgG1-F56	9.4E-07	I332V/M428L/N434Y

Table 5-2 is a continuation of Table 5-1.

[Table 5-2]

IgG1-F57	1.1E-05	G385D/Q386P/N389S
IgG1-F58	7.7E-07	G385D/Q386P/N389S/N434W
IgG1-F59	2.4E-06	G385D/Q386P/N389S/N434Y
IgG1-F60	1.1E-05	G385H
IgG1-F61	9.7E-07	G385H/N434W
IgG1-F62	1.9E-06	G385H/N434Y
IgG1-F63	2.5E-06	N434F
IgG1-F64	5.3E-06	N434H
IgG1-F65	2.9E-07	M252Y/S254T/T256E/N434F
IgG1-F66	4.3E-07	M252Y/S254T/T256E/N434H
IgG1-F67	6.3E-07	M252Y/N434F
IgG1-F68	9.3E-07	M252Y/N434H
IgG1-F69	5.1E-07	M428L/N434W
IgG1-F70	1.5E-06	M428L/N434Y
IgG1-F71	8.3E-08	M252Y/S254T/T256E/M428L/N434W
IgG1-F72	2.0E-07	M252Y/S254T/T256E/M428L/N434Y
IgG1-F73	1.7E-07	M252Y/M428L/N434W
IgG1-F74	4.6E-07	M252Y/M428L/N434Y
IgG1-F75	1.4E-06	M252Y/M428L/N434A
IgG1-F76	1.0E-06	M252Y/S254T/T256E/M428L/N434A
IgG1-F77	9.9E-07	T256E/M428L/N434Y
IgG1-F78	7.8E-07	S254T/M428L/N434W
IgG1-F79	5.9E-06	S254T/T256E/N434A
IgG1-F80	2.7E-06	M252Y/T256Q/N434A
IgG1-F81	1.6E-06	M252Y/T256E/N434A
IgG1-F82	1.1E-06	T256Q/N434W
IgG1-F83	2.6E-06	T256Q/N434Y
IgG1-F84	2.8E-07	M252W/T256Q/N434W
IgG1-F85	5.5E-07	M252W/T256Q/N434Y
IgG1-F86	1.5E-06	S254T/T256Q/N434W
IgG1-F87	4.3E-06	S254T/T256Q/N434Y
IgG1-F88	1.9E-07	M252Y/S254T/T256Q/N434W
IgG1-F89	3.6E-07	M252Y/S254T/T256Q/N434Y
IgG1-F90	1.9E-08	M252Y/T256E/V308P/N434W
IgG1-F91	4.8E-08	M252Y/V308P/M428L/N434Y
IgG1-F92	1.1E-08	M252Y/S254T/T256E/V308P/M428L/N434W
IgG1-F93	7.4E-07	M252W/M428L/N434W
IgG1-F94	3.7E-07	P257L/M428L/N434Y
IgG1-F95	2.6E-07	M252Y/S254T/T256E/M428L/N434F
IgG1-F99	6.2E-07	M252Y/T256E/N434H

[Table 6-1]

VARIANT NAME	KD (M)	AMINO ACID ALTERATION
IgG1	ND	NONE
IgG1-v1	3.2E-06	M252Y/S254T/T256E
IgG1-v2	8.1E-07	N434W
IgG1-F3	2.5E-06	N434Y
IgG1-F4	5.8E-06	N434S
IgG1-F5	6.8E-06	N434A
IgG1-F7	5.6E-06	M252Y
IgG1-F8	4.2E-06	M252W
IgG1-F9	1.4E-07	M252Y/S254T/T256E/N434Y
IgG1-F10	6.9E-08	M252Y/S254T/T256E/N434W
IgG1-F11	3.1E-07	M252Y/N434Y
IgG1-F12	1.7E-07	M252Y/N434W
IgG1-F13	3.2E-07	M252W/N434Y
IgG1-F14	1.8E-07	M252W/N434W
IgG1-F19	4.6E-07	P257L/N434Y
IgG1-F20	4.6E-07	V308F/N434Y
IgG1-F21	3.0E-08	M252Y/V308P/N434Y
IgG1-F22	2.0E-06	M428L/N434S
IgG1-F25	9.2E-09	M252Y/S254T/T256E/V308P/N434W
IgG1-F26	1.0E-06	I332V
IgG1-F27	7.4E-06	G237M
IgG1-F29	1.4E-06	I332V/N434Y
IgG1-F31	2.8E-06	G237M/V308F
IgG1-F32	8.0E-07	S254T/N434W
IgG1-F33	2.3E-06	S254T/N434Y
IgG1-F34	2.8E-07	T256E/N434W
IgG1-F35	8.4E-07	T256E/N434Y
IgG1-F36	3.6E-07	S254T/T256E/N434W
IgG1-F37	1.1E-06	S254T/T256E/N434Y
IgG1-F38	1.0E-07	M252Y/S254T/N434W
IgG1-F39	3.0E-07	M252Y/S254T/N434Y
IgG1-F40	8.2E-08	M252Y/T256E/N434W
IgG1-F41	1.5E-07	M252Y/T256E/N434Y
IgG1-F42	1.0E-06	M252Y/S254T/T256E/N434A
IgG1-F43	1.7E-06	M252Y/N434A
IgG1-F44	1.1E-06	M252W/N434A
IgG1-F47	2.4E-07	M252Y/T256Q/N434W
IgG1-F48	3.2E-07	M252Y/T256Q/N434Y
IgG1-F49	5.1E-07	M252F/T256D/N434W
IgG1-F50	1.2E-06	M252F/T256D/N434Y
IgG1-F51	8.1E-06	N434F/Y436H
IgG1-F52	3.1E-06	H433K/N434F/Y436H
IgG1-F53	1.0E-06	I332V/N434W
IgG1-F54	8.4E-08	V308P/N434W
IgG1-F56	9.4E-07	I332V/M428L/N434Y
IgG1-F57	1.1E-05	G385D/Q386P/N389S
IgG1-F58	7.7E-07	G385D/Q386P/N389S/N434W
IgG1-F59	2.4E-06	G385D/Q386P/N389S/N434Y
IgG1-F60	1.1E-05	G385H
IgG1-F61	9.7E-07	G385H/N434W
IgG1-F62	1.9E-06	G385H/N434Y
IgG1-F63	2.5E-06	N434F
IgG1-F64	5.3E-06	N434H

Table 6-2 is a continuation of Table 6-1.

[Table 6-2]

IgG1-F65	2.9E-07	M252Y/S254T/T256E/N434F
IgG1-F66	4.3E-07	M252Y/S254T/T256E/N434H
IgG1-F67	6.3E-07	M252Y/N434F
IgG1-F68	9.3E-07	M252Y/N434H
IgG1-F69	5.1E-07	M428L/N434W
IgG1-F70	1.5E-06	M428L/N434Y
IgG1-F71	8.3E-08	M252Y/S254T/T256E/M428L/N434W
IgG1-F72	2.0E-07	M252Y/S254T/T256E/M428L/N434Y
IgG1-F73	1.7E-07	M252Y/M428L/N434W
IgG1-F74	4.6E-07	M252Y/M428L/N434Y
IgG1-F75	1.4E-06	M252Y/M428L/N434A
IgG1-F76	1.0E-06	M252Y/S254T/T256E/M428L/N434A
IgG1-F77	9.9E-07	T256E/M428L/N434Y
IgG1-F78	7.8E-07	S254T/M428L/N434W
IgG1-F79	5.9E-06	S254T/T256E/N434A
IgG1-F80	2.7E-06	M252Y/T256Q/N434A
IgG1-F81	1.6E-06	M252Y/T256E/N434A
IgG1-F82	1.1E-06	T256Q/N434W
IgG1-F83	2.6E-06	T256Q/N434Y
IgG1-F84	2.8E-07	M252W/T256Q/N434W
IgG1-F85	5.5E-07	M252W/T256Q/N434Y
IgG1-F86	1.5E-06	S254T/T256Q/N434W
IgG1-F87	4.3E-06	S254T/T256Q/N434Y
IgG1-F88	1.9E-07	M252Y/S254T/T256Q/N434W
IgG1-F89	3.6E-07	M252Y/S254T/T256Q/N434Y
IgG1-F90	1.9E-08	M252Y/T256E/V308P/N434W
IgG1-F91	4.8E-08	M252Y/V308P/M428L/N434Y
IgG1-F92	1.1E-08	M252Y/S254T/T256E/V308P/M428L/N434W
IgG1-F93	7.4E-07	M252W/M428L/N434W
IgG1-F94	3.7E-07	P257L/M428L/N434Y
IgG1-F95	2.6E-07	M252Y/S254T/T256E/M428L/N434F
IgG1-F99	6.2E-07	M252Y/T256E/N434H

Such amino acid alterations can be appropriately introduced using known methods. For example, alterations in the Fc domain of human natural IgG1 are described in Drug Metab Dispos. 2007 Jan. 35(1): 86-94; Int Immunol. 2006 Dec. 18, (12): 1759-69; J Biol Chem. 2001 Mar. 2, 276(9): 6591-604; J Biol Chem. (2007) 282(3): 1709-17; J Immunol. (2002) 169(9): 5171-80; J Immunol. (2009) 182(12): 7663-71; Molecular Cell, Vol. 7, 867-877, April, 2001; Nat Biotechnol. 1997 Jul. 15, (7): 637-40; Nat Biotechnol. 2005 Oct. 23, (10): 1283-8; Proc Natl Acad Sci U S A. 2006 Dec. 5, 103(49): 18709-14; EP 2154157; US 20070141052; WO 2000/042072; WO 2002/060919; WO 2006/020114; WO 2006/031370; WO 2010/033279; WO 2006/053301; and WO 2009/086320.

According to the Journal of Immunology (2009) 182: 7663-7671, the human FcRn-

binding activity of human natural IgG1 in the acidic pH range (pH 6.0) is KD 1.7 μM, and the activity is almost undetectable in the neutral pH range. Thus, in a preferred embodiment, the antigen-binding molecule to be used in the methods of the present invention includes antigen-binding molecules whose human FcRn-binding activity in the acidic pH range is KD 20 μM or stronger, and is identical to or stronger than that of human natural IgG1 in the neutral pH range. In a more preferred embodiment, the antigen-binding molecule includes antigen-binding molecules whose human FcRn-binding activity is KD 2.0 μM or stronger in the acidic pH range and KD 40 μM or stronger in the neutral pH range. In a still more preferred embodiment, the antigen-binding molecule includes antigen-binding molecules whose human FcRn-binding activity is KD 0.5 μM or stronger in the acidic pH range and KD 15 μM or stronger in the neutral pH range. Specifically, it is preferred that the antigen-binding activity is lower under an acidic pH condition than under a neutral pH condition. The above KD values are determined by the method described in the Journal of Immunology (2009) 182: 7663-7671 (by immobilizing the antigen-binding molecule onto a chip and loading human FcRn as an analyte).

Dissociation constant (KD) can be used as a value of human FcRn-binding activity. However, human natural IgG1 has little human FcRn-binding activity in the neutral pH range (pH 7.4), and therefore it is difficult to calculate the activity as KD. Methods for assessing whether the human FcRn-binding activity is higher than that of human natural IgG1 at pH 7.4 include assessment methods by comparing the intensities of Biacore response after loading analytes at the same concentration. Specifically, when the response after loading a human FcRn chip immobilized with an antigen-binding molecule at pH 7.4 is stronger than the response after loading human FcRn onto a chip immobilized with human natural IgG1 at pH 7.4, the human FcRn-binding activity of the antigen-binding molecule is judged to be higher than that of human natural IgG1 at pH 7.4.

pH 7.0 can also be used as a neutral pH range. Using pH 7.0 as a neutral pH can facilitate weak interaction between human FcRn and FcRn-binding domain. As a temperature employed in the assay condition, a binding affinity may be assessed at any temperature from 10°C to 50°C. Preferably, a temperature at from 15°C to 40°C is employed in order to determine the binding affinity between human FcRn-binding domain and human FcRn. More preferably, any temperature at from 20°C to 35°C, like any one of 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, and 35°C is also employed in order to determine the binding affinity between human FcRn-binding domain and human FcRn. A temperature at 25°C described in Example 5 is one of example for the embodiment of this invention. In a preferred embodiment, an interaction between human FcRn and FcRn-binding domain can be measured at pH 7.0 and at 25°C as described in Example 5. Binding affinity of antigen-binding molecule to human FcRn can be measured by Biacore as described in Example 3.

In a more preferred embodiment, the antigen-binding molecules of the present invention have human FcRn-binding activity at pH 7.0 and at 25°C which is stronger than natural human IgG. In a more preferred embodiment, human FcRn-binding activity at pH 7.0 and at 25°C is 28-fold stronger than natural human IgG or stronger than KD 3.2 μM. In a more preferred embodiment, human FcRn-binding activity at pH 7.0 and at 25°C is 38-fold stronger than natural human IgG or stronger than KD 2.3 μM.

A natural human IgG1, IgG2, IgG3 or IgG4 is preferably used as the intact human IgG for a purpose of a reference intact human IgG to be compared with the antigen-binding molecules for their human FcRn binding activity or *in vivo* binding activity. Preferably, a reference antigen-binding molecule comprising the same antigen-binding domain as an antigen-binding molecule of the interest and natural human IgG Fc region as a human FcRn-binding domain can be appropriately used. More preferably, a natural human IgG1 is used for a purpose of a reference natural human IgG to be compared with the antigen-binding molecules for their human FcRn binding activity or *in vivo* binding activity.

More specifically, the antigen-binding molecules with long term effect on activity for eliminating antigen in plasma described in the present invention have human FcRn-binding activity at pH 7.0 and at 25°C within a range of 28-fold to 440-fold stronger than natural human IgG1 or KD within a range of 3.0 μM to 0.2 μM. A long term plasma antigen concentration is determined by measuring total or free antigen concentration in plasma and molar antigen/antigen-binding molecule ratio at 2, 4, 7, 14, 28, 56, or 84 days after administration of an antigen-binding molecule in order to evaluate the long term effect of the antigen-binding molecule of the present invention on activity for eliminating antigen in plasma. Whether the reduction of plasma antigen concentration or molar antigen/antigen-binding molecule ratio is achieved by antigen-binding molecule described in the present invention can be determined by the evaluation of the reduction at any one or more of the time points described above.

Still more specifically, the antigen-binding molecules with short term effect on activity for eliminating antigen in plasma described in the present invention have human FcRn-binding activity at pH 7.0 and at 25°C 440-fold stronger than natural human IgG or KD stronger than 0.2 μM. A short term plasma antigen concentration is determined by measuring total or free antigen concentration in plasma and molar antigen/antigen-binding molecule ratio at 15 min, 1, 2, 4, 8, 12, or 24 hours after administration of an antigen-binding molecule in order to evaluate the short term effect of the antigen-binding molecule of the present invention on activity for eliminating antigen in plasma.

The methods of the present invention are applicable to any antigen-binding molecules regardless of the type of target antigen.

For example, when the antigen-binding molecule is an antibody that binds to a membrane antigen, the antibody administered into the body binds to the antigen and then is taken up via internalization into endosomes in the cells together with the antigen while the antibody is kept bound to the antigen. Then, the antibody translocates to lysosomes while the antibody is kept bound to the antigen, and the antibody is degraded by the lysosome together with the antigen. The internalization-mediated elimination from the plasma is called antigen-dependent elimination, and such elimination has been reported with numerous antibody molecules (Drug Discov Today. 2006 Jan; 11(1-2): 81-8). When a single molecule of IgG antibody binds to antigens in a divalent manner, the single antibody molecule is internalized while the antibody is kept bound to the two antigen molecules, and degraded in the lysosome. Accordingly, in the case of common antibodies, one molecule of IgG antibody cannot bind to three or more molecules of antigen. For example, a single IgG antibody molecule having a neutralizing activity cannot neutralize three or more antigen molecules.

The relatively prolonged retention (slow elimination) of IgG molecules in the plasma is due to the function of human FcRn which is known as a salvage receptor of IgG molecules. When taken up into endosomes *via* pinocytosis, IgG molecules bind to human FcRn expressed in the endosomes under the acidic condition in the endosomes. While IgG molecules that did not bind to human FcRn transfer to lysosomes where they are degraded, IgG molecules that are bound to human FcRn translocate to the cell surface and return again in the plasma by dissociating from human FcRn under the neutral condition in the plasma.

Alternatively, when the antigen-binding molecule is an antibody that binds to a soluble antigen, the antibody administered into the body binds to the antigen and then is taken up into cells while the antibody is kept bound to the antigen. Many antibodies taken up into cells are released to the outside of the cell *via* FcRn. However, since the antibodies are released to the outside of the cell, with the antibodies kept bound to antigens, the antibodies cannot bind to antigens again. Thus, similar to antibodies that bind to membrane antigens, in the case of common antibodies, one molecule of IgG antibody cannot bind to three or more antigen molecules.

Calcium concentration-dependent antigen-binding antibodies that strongly bind to an antigen under high calcium concentration conditions in plasma but dissociate from the antigen under low calcium concentration conditions in the endosome can dissociate from the antigen in the endosome. Such calcium concentration-dependent antigen-binding antibodies can bind to antigens again when they are recycled to the plasma by FcRn after antigen dissociation; thus, each antibody can repeatedly bind to a number of antigens. Furthermore, the antigen bound to the antigen-binding molecule is dissociated in the endosome and not recycled to the plasma. This facilitates the antigen-binding molecule-mediated antigen uptake into cells. Thus, the

administration of an antigen-binding molecule can enhance the antigen elimination and thereby reduces the plasma antigen concentration.

Antigen-binding molecules

The present invention provides antigen-binding molecules having an antigen-binding domain and a human FcRn-binding domain, wherein the antigen-binding activity of the antigen-binding molecules is different under two different calcium concentration conditions and is lower under a low calcium concentration condition than under a high calcium concentration condition.

The antigen-binding molecules of the present invention are not particularly limited, as long as they include an antigen-binding domain having a binding activity specific to a target antigen. Such preferred antigen-binding domains comprise, for example, domains having an antigen-binding region of an antibody. The antigen-binding region of an antibody comprises, for example, CDRs and variable regions. When the antigen-binding region of an antibody is CDR, it may contain all six CDRs from the whole antibody, or one, two, or more CDRs. When CDRs are contained as a binding region in an antibody, they may comprise amino acid deletions, substitutions, additions, and/or insertions, or may be a portion of a CDR.

On the other hand, antigen-binding molecules to be used in the methods of the present invention include antigen-binding molecules that have an antagonistic activity (antagonistic antigen-binding molecules), antigen-binding molecules that have an agonistic activity (agonistic antigen-binding molecule), and molecules having cytotoxicity. In a preferred embodiment, the antigen-binding molecules comprise antagonistic antigen-binding molecules, in particular, antagonistic antigen-binding molecules that recognize an antigen such as a receptor or cytokine.

In the present invention, the antigen-binding molecule of interest is not particularly limited, and may be any antigen-binding molecules. The antigen-binding molecule of the present invention preferably has both an antigen-binding activity (antigen-binding domain) and a human FcRn-binding domain. In particular, a preferred antigen-binding molecule of the present invention comprises a human FcRn-binding domain.

The antigen-binding molecule comprising both an antigen-binding domain and a human FcRn-binding domain includes, for example, antibodies. In the context of the present invention, a preferred example of antibody includes IgG antibodies. When the antibody to be used is an IgG antibody, the type of IgG is not limited; and an IgG belonging to any isotype (subclass) such as IgG1, IgG2, IgG3, or IgG4 can be used. Furthermore, the antigen-binding molecules of the present invention may comprise an antibody constant region, and amino acid mutations may be introduced into the constant region. Amino acid mutations to be introduced include, for example, those that potentiate or impair the binding to Fcγ receptor (Proc Natl Acad Sci U S A. 2006 Mar 14; 103(11): 4005-10), but are not limited to these examples. Alternatively, it is also possible to

alter the pH-dependent binding by selecting an appropriate constant region such as that of IgG2.

When the antigen-binding molecule of interest in the present invention is an antibody, it may be an antibody derived from any animal, such as a mouse antibody, human antibody, rat antibody, rabbit antibody, goat antibody, or camel antibody. Furthermore, the antibody may be an altered antibody, for example, a chimeric antibody, and in particular, an altered antibody including amino acid substitutions in the sequence of a humanized antibody, and such. The antibodies also include bispecific antibodies, antibody modification products linked with various molecules, and polypeptides comprising antibody fragments.

"Chimeric antibodies" are antibodies prepared by combining sequences derived from different animals. Specifically, the chimeric antibody includes, for example, antibodies having heavy and light chain variable (V) regions from a mouse antibody and heavy and light chain constant (C) regions from a human antibody.

"Humanized antibodies", also referred to as reshaped human antibodies, are antibodies in which the complementarity determining regions (CDRs) of an antibody derived from a nonhuman mammal, for example, a mouse, are transplanted into the CDRs of a human antibody. Methods for identifying CDRs are known (Kabat et al., Sequence of Proteins of Immunological Interest (1987), National Institute of Health, Bethesda, Md.; Chothia et al., Nature (1989) 342: 877). General genetic recombination technologies suitable for this purpose are also known (see European Patent Application EP 125023; and WO 96/02576).

A bispecific antibody refers to an antibody that has variable regions in the same antibody molecule that recognize different epitopes. A bispecific antibody may be an antibody that recognizes two or more different antigens, or an antibody that recognizes two or more different epitopes on a same antigen.

Furthermore, polypeptides comprising antibody fragments include, for example, Fab fragments, F(ab')2 fragments, scFvs (Nat Biotechnol. 2005 Sep; 23(9): 1126-36), domain antibodies (dAbs) (WO 2004/058821; WO 2003/002609), scFv-Fc (WO 2005/037989), dAb-Fc, and Fc fusion proteins. The Fc region of a molecule comprising Fc region can be used as a human FcRn-binding domain. Alternatively, an FcRn-binding domain may be fused to these molecules.

Further, antigen-binding molecules that are applicable to the present invention may be antibody-like molecules. An antibody-like molecule (scaffold molecule, peptide molecule) is a molecule that can exhibit functions by binding to a target molecule (Current Opinion in Biotechnology (2006) 17: 653-658; Current Opinion in Biotechnology (2007) 18: 1-10; Current Opinion in Structural Biology (1997) 7: 463-469; Protein Science (2006) 15: 14-27), and includes, for example, DARPins (WO 2002/020565), Affibody (WO 1995/001937), Avimer (WO 2004/044011; WO 2005/040229), and Adnectin (WO 2002/032925). These antibody-like

molecules can bind to target molecules in a calcium concentration-dependent manner, facilitate antigen uptake into cells by antigen-binding molecules, facilitate reduction of plasma antigen concentration by administering antigen-binding molecules, and improve plasma retention of antigen-binding molecules, and increase the number of times of antigen binding by a single antigen-binding molecule.

Furthermore, the antigen-binding molecule may be a protein resulting from fusion between a human FcRn-binding domain and a receptor protein that binds to a target, and includes, for example, TNFR-Fc fusion proteins, IL1R-Fc fusion proteins, VEGFR-Fc fusion proteins, and CTLA4-Fc fusion proteins (Nat Med. 2003, Jan; 9(1): 47-52; BioDrugs. (2006) 20(3): 151-60). If these fusion proteins of receptor and human FcRn-binding domain bind to a target molecule in a calcium concentration-dependent manner, it is possible to facilitate antigen uptake into cells by antigen-binding molecules, facilitate the reduction of plasma antigen concentration by administering antigen-binding molecules, and improve plasma retention of the antigen-binding molecules, and increase the number of times of antigen binding by a single antigen-binding molecule.

Moreover, the antigen-binding molecule may be a fusion protein between an artificial ligand protein that binds to a target and has a neutralizing effect and a human FcRn-binding domain; and an artificial ligand protein includes, for example, mutant IL-6 (EMBO J. 1994 Dec 15; 13(24): 5863-70). If such artificial ligand fusion proteins can bind to target molecules in a calcium concentration-dependent manner, it is possible to facilitate antigen uptake into cells by antigen-binding molecules, facilitate reduction of plasma antigen concentration by administering antigen-binding molecules, improve plasma retention of antigen-binding molecules, and increase the number of times of antigen binding by a single antigen-binding molecule.

Furthermore, sugar chains may be modified in the antibodies of the present invention. Antibodies with altered sugar chains include, for example, antibodies with modified glycosylation (WO 99/54342 and such), antibodies that are deficient in sugar chain-attached fucose (WO 00/61739; WO 02/31140; WO 2006/067847; WO 2006/067913), and antibodies having sugar chains with bisecting GlcNAc (WO 02/79255).

Besides ionized calcium concentration, conditions used for measuring antigen-binding activity can be appropriately selected by those skilled in the art, and they are not particularly limited. For example, the conditions of using HEPES buffer at 37°C may be used to determine the activity. For example, Biacore (GE Healthcare) or such can be used to determine the activity. When the antigen is a soluble antigen, the activity of an antigen-binding molecule to bind to the soluble antigen can be determined by loading the antigen as an analyte onto a chip immobilized with the antigen-binding molecule. Alternatively, when the antigen is a membrane-type antigen, the activity of the antigen-binding molecule to bind to the membrane-type antigen can be

determined by loading the antigen-binding molecule as an analyte onto an antigen-immobilized chip.

In the antigen-binding molecules of the present invention, the ratio of antigen-binding activity under a low calcium concentration condition to that under a high calcium concentration condition is not particularly limited as long as the antigen-binding activity is lower under the low calcium concentration condition than under the high calcium concentration condition. However, the value of KD (Ca 3 μM)/KD (Ca 2 mM), which is a ratio of dissociation constant (KD) against an antigen under a low calcium concentration condition to that under a high calcium concentration condition, is preferably 2 or greater, more preferably 10 or greater, and still more preferably 40 or greater. The upper limit of the KD (Ca 3 μM)/KD (Ca 2 mM) value is not particularly limited, and may be any value, for example, 400, 1,000, or 10,000, as long as production is possible by using the technologies of those skilled in the art.

When the antigen is a soluble antigen, the value of antigen-binding activity can be presented in terms of the dissociation constant (KD). On the other hand, when the antigen is a membrane-type antigen, the activity can be presented in terms of apparent dissociation constant (apparent KD). The dissociation constant (KD) and apparent dissociation constant (apparent KD) can be determined by methods known to those skilled in the art, for example, using Biacore (GE Healthcare), Scatchard plot, flow cytometer, or such.

In the antigen-binding molecules of the present invention, other parameters that are representative of the ratio between the antigen-binding activities under a low calcium concentration condition and a high calcium concentration condition include, for example, dissociation rate constant k_d. When the dissociation rate constant (k_d) is used instead of the dissociation constant (KD) as a parameter representative of the binding activity ratio, the value of k_d (under a low calcium concentration condition)/k_d (under a high calcium concentration condition), which is a ratio between the k_d (dissociation rate constant) values against an antigen under a low calcium concentration condition and a high calcium concentration condition, is preferably 2 or greater, more preferably 5 or greater, even more preferably 10 or greater, and still more preferably 30 or greater. The upper limit of the k_d (under the condition of low calcium concentration)/k_d (under condition of high calcium condition) value is not particularly limited, and may be any value, for example, 50, 100, or 200, as long as production is possible by using the technologies of those skilled in the art.

When the antigen is a soluble antigen, the value of antigen-binding activity can be presented using the dissociation rate constant (k_d). Alternatively, when the antigen is a membrane-type antigen, the value can be presented in terms of apparent k_d (apparent dissociation rate constant). The dissociation rate constant (k_d) and apparent dissociation rate constant (apparent k_d) can be determined by methods known to those skilled in the art, for example, using

Biacore (GE Healthcare), flow cytometer, or the like.

In the present invention, when measuring the antigen-binding activity of an antigen-binding molecule at a different calcium concentration, it is preferable to use the same conditions except for the calcium concentration.

There is no particular limitation on the method for reducing (weakening) the antigen-binding activity of an antigen-binding molecule under a low calcium concentration condition to be lower than that under a high calcium concentration condition (method for conferring a calcium concentration-dependent antigen-binding activity) in order to obtain an antigen-binding molecule that has a lower antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition. Antigen-binding molecules that have a lower (weaker) antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition (antigen-binding molecules that show calcium concentration-dependent binding) can be obtained directly, for example, by screening an *in vitro*-displayed antibody library using the above-mentioned calcium concentration-dependent binding to an antigen as an indicator.

Other methods include methods for directly isolating an antigen-binding molecule having the above-mentioned property. For example, it is possible to directly obtain an antibody having a property of interest by immunizing animals (mice, rats, hamsters, rabbits, human immunoglobulin transgenic mice, human immunoglobulin transgenic rats, human immunoglobulin transgenic rabbits, llamas, camels, etc.) with an antigen, and screening the obtained antibodies using the calcium concentration-dependent antigen binding as an indicator. Alternatively, random mutations may be introduced into the amino acid sequence of an antigen-binding molecule, and the antigen-binding activity of the antigen-binding molecule at different calcium concentration conditions is measured by the above-mentioned method to select an antigen-binding molecule that has a lower antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition in comparison to the antigen-binding molecule before modification.

When the antigen-binding activity of an antigen-binding molecule under a low calcium concentration condition is reduced (weakened) to be lower than that under a high calcium concentration condition (the value of KD (under a low calcium concentration condition)/KD (under a high calcium concentration condition) is increased) by the above-mentioned method or such, the value of KD (under a low calcium concentration condition)/KD (under a high calcium concentration condition) is, without particular limitation, typically twice or more, preferably five times or more, and more preferably ten times or more in comparison to the original antibody.

Furthermore, by using a method for conferring the calcium concentration-dependent antigen-binding activity of the present invention, in combination with a method of using an

antigen-binding molecule having human FcRn-binding activity at neutral pH or a method of conferring or increasing the human FcRn-binding activity at neutral pH, it is possible to enhance the function of promoting antigen uptake into cells, function of increasing the number of times of antigen-binding by one antigen-binding molecule, function of promoting the reduction of plasma antigen concentration by administering an antigen-binding molecule, or function of improving the plasma retention of an antigen-binding molecule. The methods of conferring or increasing the human FcRn-binding activity at neutral pH include, for example, the above-described methods for modifying amino acids in the human-FcRn-binding domain. Herein, "human FcRn-binding activity at neutral pH" means the activity to bind to human FcRn at pH 6.7 to 10.0. A preferable human FcRn-binding activity is, for example, the human FcRn-binding activity at pH 7.0 to 8.0; and a more preferable human FcRn-binding activity is, for example, the human FcRn-binding activity at pH 7.4.

Furthermore, by using a method for conferring the calcium concentration-dependent antigen-binding activity of the present invention, in combination with a method of using an antigen-binding molecule having pH-dependent antigen-binding activity or a method of conferring a pH-dependent antigen-binding activity, it is possible to enhance the function of promoting antigen uptake into cells, function of increasing the number of times of antigen-binding by one antigen-binding molecule, function of promoting the reduction of plasma antigen concentration by administering an antigen-binding molecule, or function of improving the plasma retention of an antigen-binding molecule. The methods of conferring a pH-dependent antigen-binding activity include, for example, methods described in WO 2009/125825.

Specifically, for example, a calcium concentration-dependent antigen-binding molecule of the present invention can be used in combination with a method for reducing (weakening) the antigen-binding activity of an antigen-binding molecule at acidic pH to be lower than that at neural pH. Herein, "reducing (weakening) the antigen-binding activity at acidic pH to be lower than the antigen-binding activity at neural pH" means reducing the antigen-binding activity of an antigen-binding molecule at pH 4.0 to 6.5 to be lower than that at pH 6.7 to 10.0. It preferably means weakening the antigen-binding activity of an antigen-binding molecule at pH 5.5 to 6.5 to be lower than that at pH 7.0 to 8.0, and particularly preferably means weakening the antigen-binding activity of an antigen-binding molecule at pH 5.8 to be lower than that at pH 7.4. Herein, "acidic pH" typically refers to pH 4.0 to 6.5, preferably pH 5.5 to 6.5, and particularly preferably pH 5.8. Meanwhile, herein "neutral pH" typically refers to pH 6.7 to 10.0, preferably pH 7.0 to 8.0, and particularly preferably pH 7.4.

On the other hand, the phrase "reducing the antigen-binding activity of an antigen-binding molecule at acidic pH to be lower than that at neutral pH" is synonymous with "increasing the antigen-binding activity of an antigen-binding molecule at neutral pH to be

greater than that at acidic pH". Specifically, in the present invention, one may increase the difference between the antigen-binding activities of an antigen-binding molecule at acidic pH and neutral pH (for example, one may increase the value of KD (pH5.8)/KD (pH7.4) as described below). The difference between the antigen-binding activities of an antigen-binding molecule at acidic pH and neutral pH may be increased, for example, by reducing the antigen-binding activity at acidic pH, or increasing the antigen-binding activity at neutral pH, or both.

In the present invention, the difference between the antigen-binding activities at acidic pH and neutral pH is not particularly limited as long as the antigen-binding activity is lower at acidic pH than at neutral pH. However, the value of KD (pH 5.8)/KD (pH 7.4), which is a ratio between the dissociation constants (KD) against an antigen at pH 5.8 and pH 7.4, is preferably 2 or greater, more preferably 10 or greater, and still more preferably 40 or greater. The upper limit of the KD (pH 5.8)/KD (pH 7.4) value is not particularly limited, and may be any value, for example, 400, 1,000, or 10,000, as long as production is possible by using the technologies of those skilled in the art.

In the present invention, other parameters that are representative of the ratio between antigen-binding activities at acidic pH and neutral pH include, for example, dissociation rate constant k_d. When the dissociation rate constant (k_d) is used instead of the dissociation constant (KD) as a parameter representative of the binding activity ratio, the value of k_d (pH 5.8)/k_d (pH 7.4), which is a ratio between the k_d (dissociation rate constant) values against an antigen at pH 5.7 and pH 7.4, is preferably 2 or greater, more preferably 5 or greater, even more preferably 10 or greater, and still more preferably 30 or greater. The upper limit of the k_d (pH 5.8)/k_d (pH 7.4) value is not particularly limited, and may be any value, for example, 50, 100, or 200, as long as production is possible by using the technologies of those skilled in the art.

The methods for conferring a pH-dependent antigen-binding activity are not particularly limited. Such methods include, for example, methods for weakening the antigen-binding activity at pH 5.8 to be lower than that at pH 7.4 by substituting at least one amino acid in an antigen-binding molecule with histidine, or inserting at least one histidine into an antigen-binding molecule. It is already known that substitution of an amino acid in an antibody with histidine can confer a pH-dependent antigen-binding activity to the antibody (FEBS Letter, 309(1): 85-88, (1992)). In the present invention, sites of histidine mutation (substitution) or insertion in an antigen-binding molecule are not particularly limited, and any site can be used as long as the antigen-binding activity at pH 5.8 becomes weaker than that at pH 7.4 (the value of KD (pH5.8)/KD (pH7.4) becomes greater) in comparison to before the mutation or insertion. For example, when the antigen-binding molecule is an antibody, such sites include an antibody variable region. The number of histidine mutation or insertion sites introduced (or made) can be appropriately determined by those skilled in the art. Only one site may be substituted with

histidine, or histidine may be inserted at only one site. Alternatively, two or more multiple sites may be substituted with histidine, or histidine may be inserted at two or more multiple sites. It is also possible to introduce a mutation besides histidine mutation (mutation into an amino acid besides histidine) at the same time. Furthermore, histidine mutation may be introduced simultaneously with histidine insertion. It is possible to substitute or insert histidine at random using a method such as histidine scanning, which uses histidine instead of alanine in alanine scanning known to those skilled in the art. Alternatively, an antigen-binding molecule whose KD (pH 5.8)/KD (pH 7.4) is increased compared to before mutation can be selected from a library of antigen-binding molecules into which a random histidine mutation or insertion has been introduced.

When at least one amino acid in an antigen-binding molecule is substituted with histidine, or at least one histidine is inserted into the amino acids of an antigen-binding molecule, while there is no particular limitation, it is preferred that the antigen-binding activity of the antigen-binding molecule at pH 7.4 after histidine substitution or insertion is comparable to that at pH 7.4 before histidine substitution or insertion. Herein, the phrase "the antigen-binding activity of an antigen-binding molecule at pH 7.4 after histidine substitution or insertion is comparable to that at pH 7.4 before histidine substitution or insertion" means that the antigen-binding molecule after histidine substitution or insertion retains 10% or more, preferably 50% or more, more preferably 80% or more, and still more preferably 90% or more of the antigen-binding activity before histidine substitution or insertion. When the antigen-binding activity of an antigen-binding molecule is impaired by a histidine substitution or insertion, the antigen-binding activity may be made to be comparable to that before the histidine substitution or insertion by introducing one or more amino acid substitutions, deletions, additions, and/or insertions into the antigen-binding molecule. The present invention also includes antigen-binding molecules having a comparable binding activity made by one or more amino acid substitutions, deletions, additions, and/or insertions after histidine substitution or insertion.

Alternative methods for weakening the antigen-binding activity of an antigen-binding molecule at pH 5.8 to be lower than that at pH 7.4 include methods of substituting an amino acid in an antigen-binding molecule with a non-natural amino acid, or inserting a non-natural amino acid into the amino acids of an antigen-binding molecule. It is known that pKa can be artificially controlled using non-natural amino acids (Angew. Chem. Int. Ed. 2005, 44, 34; Chem Soc Rev. 2004 Sep. 10; 33(7): 422-30; Amino Acids. 1999; 16(3-4): 345-79). Thus, in the present invention, non-natural amino acids can be used instead of histidine mentioned above. Substitution and/or insertion of a non-natural amino acid may be introduced simultaneously with the above-mentioned histidine substitution and/or insertion. Any non-natural amino acids may be used in the present invention. It is possible to use non-natural amino acids or such known to

those skilled in the art.

Furthermore, when the antigen-binding molecule is a substance containing an antibody constant region, alternative methods for weakening the antigen-binding activity of the antigen-binding molecule at pH 5.8 to be lower than that at pH 7.4 include methods for modifying the antibody constant region contained in the antigen-binding molecule. Examples of modifying an antibody constant region include methods for substituting a constant region described in WO 2009/125825.

Meanwhile, methods for altering an antibody constant region include, for example, methods for assessing various constant region isotypes (IgG1, IgG2, IgG3, and IgG4) and selecting isotypes that reduce the antigen-binding activity at pH 5.8 (increase the dissociation rate at pH 5.8). Such methods also include methods for reducing the antigen-binding activity at pH 5.8 (increasing the dissociation rate at pH 5.8) by introducing amino acid substitutions into the amino acid sequences of wild-type isotypes (amino acid sequences of wild type IgG1, IgG2, IgG3, or IgG4). The sequence of hinge region in the antibody constant region is considerably different among isotypes (IgG1, IgG2, IgG3, and IgG4), and the difference in the hinge region amino acid sequence has a great impact on the antigen-binding activity. Thus, it is possible to select an appropriate isotype to reduce the antigen-binding activity pH 5.8 (increase the dissociation rate at pH 5.8) according to the type of antigen or epitope. Furthermore, since the difference in the hinge region amino acid sequence has a great impact on the antigen-binding activity, preferred amino acid substitution sites in the amino acid sequences of wild-type isotypes are assumed to be within the hinge region.

When the antigen-binding activity of an antigen-binding substance at pH 5.8 is weakened to be lower than that at pH 7.4 (when the value of KD (pH 5.8)/KD (pH 7.4) is increased) by the above-described method and the like, it is generally preferable that the KD (pH 5.8)/KD (pH 7.4) value is twice or more, preferably five times or more, and more preferably ten times or more in comparison to the original antibody, but it is not particularly limited thereto.

Antigen-binding molecules

Furthermore, the present invention provides antigen-binding molecules whose antigen-binding activity differs at two different calcium concentration conditions; *i.e.*, the antigen-binding activity is lower under a low calcium concentration condition than under a high calcium concentration condition. Preferably, the present invention provides antigen-binding molecules that have a lower antigen-binding activity under a low calcium concentration condition (ionized calcium concentration of 0.1 μM to 30 μM) than under a high calcium concentration condition (ionized calcium concentration of 100 μM to 10 mM). More specifically, the antigen-binding molecules include antigen-binding molecules that have a lower antigen-binding activity at the

ionized calcium concentration in the early endosome *in vivo* (a low calcium concentration such as 1 μM to 5 μM) than at the ionized calcium concentration in plasma *in vivo* (a high calcium concentration such as 0.5 mM to 2.5 mM).

With respect to the antigen-binding activity of an antigen-binding molecule of the present invention that has a lower antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition, there is no limitation on the difference in antigen-binding activity as long as the antigen-binding activity is lower under a low calcium concentration condition than under a high calcium concentration condition. It is even acceptable that the antigen-binding activity of an antigen-binding molecule is only slightly lower under a low calcium concentration condition.

In a preferred embodiment, for an antigen-binding molecule of the present invention that has a lower antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition, the value of KD (low Ca)/KD (high Ca), which is the KD ratio between low and high calcium concentration conditions, is 2 or more, preferably the value of KD (low Ca)/KD (high Ca) is 10 or more, and more preferably the value of KD (low Ca)/KD (high Ca) is 40 or more. The upper limit of the KD (low Ca)/KD (high Ca) value is not particularly limited, and may be any value such as 400, 1,000, and 10,000 as long as it can be produced by techniques known to those skilled in the art.

In another preferred embodiment, for an antigen-binding molecule of the present invention that has a lower antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition, the value of k_d (low Ca)/k_d (high Ca), which is a ratio between the k_d values for an antigen at a low calcium concentration condition and high calcium concentration condition, is 2 or more, preferably the value of k_d (low Ca)/k_d (high Ca) is 5 or more, more preferably the value of k_d (low Ca)/k_d (high Ca) is 10 or more, and still more preferably the value of k_d (low Ca)/k_d (high Ca) is 30 or more. The upper limit of the k_d (low Ca)/k_d (high Ca) value is not particularly limited, and may be any value such as 50, 100, and 200 as long as it can be produced by techniques known to those skilled in the art.

An antigen-binding molecule of the present invention may additionally have the above-mentioned human FcRn-binding activity under a neutral pH condition. By using the human FcRn-binding activity under a neutral pH condition in combination with a calcium concentration-dependent antigen-binding activity, it is possible to enhance the function of promoting antigen uptake into cells, function of increasing the number of times of antigen binding by one antigen-binding molecule, function of promoting the reduction of plasma antigen concentration by administering an antigen-binding molecule, or function of improving the plasma retention of an antigen-binding molecule.

An antigen-binding molecule of the present invention may additionally have the above-

mentioned pH-dependent antigen-binding activity, *i.e.*, a lower antigen-binding activity under an acidic pH condition than under a neutral pH condition. By using the pH-dependent antigen-binding activity in combination with a calcium concentration-dependent antigen-binding activity, it is possible to enhance the function of promoting antigen uptake into cells, function of increasing the number of times of antigen binding by one antigen-binding molecule, function of promoting the reduction of plasma antigen concentration by administering an antigen-binding molecule, or function of improving the plasma retention of an antigen-binding molecule.

Furthermore, an antigen-binding molecule of the present invention may have any other property as long as it has a lower antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition. For example, the antigen-binding molecule may be an agonistic antigen-binding molecule or antagonistic antigen-binding molecule. Preferred antigen-binding molecules of the present invention include, for example, antagonistic antigen-binding molecules. Such antagonistic antigen-binding molecule is typically an antigen-binding molecule that inhibits receptor-mediated intracellular signal transduction by inhibiting the binding between a ligand (agonist) and its receptor.

Furthermore, an antigen-binding molecule to which the pH-dependent antigen-binding activity is conferred may have a substitution of histidine for at least one amino acid, or an insertion of at least one histidine.

Meanwhile, there is no particular limitation on the antigen to which an antigen-binding molecule of the present invention binds, and the antigen-binding molecule may bind to any antigen. Such antigens include, for example, membrane antigens such as receptor proteins (membrane-type receptors and soluble receptors) and cell surface markers, and soluble antigens such as cytokines. Specific examples of other antigens are described above.

Screening methods

The present invention provides methods of screening for an antigen-binding molecule that has a lower antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition. The present invention also provides methods of screening for an antigen-binding molecule having at least one function selected from:

(i) function of promoting uptake of an antigen into cells;

(ii) function of binding to an antigen two or more times;

(iii) function of promoting the reduction of plasma antigen concentration; and

(iv) function of excellence in plasma retention.

Specifically, the present invention provides methods of screening for an antigen-binding molecule, which comprises the steps of (a) to (c) below:

(a) determining the antigen-binding activity of an antigen-binding molecule under a low calcium

concentration condition;

(b) determining the antigen-binding activity of the antigen-binding molecule under a high calcium concentration condition; and

(c) selecting an antigen-binding molecule that has a lower antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition.

The present invention also provides methods of screening for an antigen-binding molecule, which comprises the steps of (a) to (c) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a high calcium concentration condition;

(b) placing an antigen-binding molecule that binds to the antigen in step (a) under a low calcium concentration condition; and

(c) obtaining an antigen-binding molecule that dissociates in step (b).

The present invention also provides methods of screening for an antigen-binding molecule, which comprises the steps of (a) to (d) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a low calcium concentration condition;

(b) selecting an antigen-binding molecule that does not bind to the antigen in step (a);

(c) allowing the antigen-binding molecule selected in step (b) to bind to the antigen under a high calcium concentration condition; and

(d) obtaining an antigen-binding molecule that binds to the antigen in step (c).

The present invention also provides methods of screening for an antigen-binding molecule, which comprises the steps of (a) to (c) below:

(a) contacting an antigen-binding molecule or a library of antigen-binding molecules with an antigen-immobilized column under a high calcium concentration condition;

(b) eluting an antigen-binding molecule that binds to the column in step (a) from the column under a low calcium concentration condition; and

(c) obtaining the antigen-binding molecule eluted in step (b).

The present invention also provides methods of screening for an antigen-binding molecule, which comprises the steps of (a) to (d) below:

(a) allowing an antigen-binding molecule or a library of antigen-binding molecules to pass through an antigen-immobilized column under a low calcium concentration condition;

(b) collecting an antigen-binding molecule eluted without binding to the column in step (a);

(c) allowing the antigen-binding molecule collected in step (b) to bind to the antigen under a high calcium concentration condition; and

(d) obtaining an antigen-binding molecule that binds to the antigen in step (c).

The present invention also provides methods of screening for an antigen-binding

molecule, which comprises the steps of (a) to (d) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a high calcium concentration condition;

(b) obtaining an antigen-binding molecule that binds to the antigen in step (a);

(c) placing the antigen-binding molecule obtained in step (b) under a low calcium concentration condition; and

(d) obtaining an antigen-binding molecule whose antigen-binding activity in step (c) is lower than an antigen-binding activity in step (b).

The above steps may be repeated two or more times. Thus, the present invention provides screening methods that further comprise the step of repeating the steps of (a) to (c), or (a) to (d) two or more times in the above-mentioned screening methods. The number of times steps (a) to (c) or (a) to (d) are repeated is not particularly limited, and it is generally ten or less.

In the screening methods of the present invention, the antigen-binding activity of an antigen-binding molecule under a low calcium concentration condition is not particularly limited, as long as it is an antigen-binding activity at an ionized calcium concentration of 0.1 μM to 30 μM. Preferably, the antigen-binding activity includes antigen-binding activities at an ionized calcium concentration of 0.5 μM to 10 μM. More preferable ionized calcium concentrations include ionized calcium concentrations in the early endosome *in vivo*. Specifically, the antigen-binding activity includes activities at 1 μM to 5 μM. Meanwhile, the antigen-binding activity of an antigen-binding molecule under a high calcium concentration condition is not particularly limited, as long as it is an antigen-binding activity at an ionized calcium concentration of 100 μM to 10 mM. Preferably, the antigen-binding activity includes antigen-binding activities at an ionized calcium concentration of 200 μM to 5 mM. More preferred ionized calcium concentrations include ionized calcium concentrations in plasma *in vivo*. Specifically, the antigen-binding activity includes activities at 0.5 mM to 2.5 mM.

The antigen-binding activity of an antigen-binding molecule can be determined by methods known to those skilled in the art. Appropriate conditions besides ionized calcium concentration can be selected by those skilled in the art. The antigen-binding activity of an antigen-binding molecule can be assessed by using KD (dissociation constant), apparent KD (apparent dissociation constant), dissociation rate k_d (dissociation rate), apparent k_d (apparent dissociation: apparent dissociation rate), or such. They can be determined by methods known to those skilled in the art, for example, using Biacore (GE Healthcare), Scatchard plot, FACS, or such.

In the present invention, the step of selecting an antigen-binding molecule that has a greater antigen-binding activity under a high calcium concentration condition than under a low calcium concentration is synonymous with the step of selecting an antigen-binding molecule that

has a lower antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition.

The difference between the antigen binding activity under a high calcium concentration condition and that under a low calcium concentration condition is not particularly limited, as long as the antigen-binding activity is greater under a high calcium concentration condition than under a low calcium concentration condition. However, the antigen-binding activity under a high calcium concentration condition is preferably twice or more, more preferably 10 times or more, and still more preferably 40 times or more of the antigen-binding activity under a low calcium concentration condition.

Antigen-binding molecules to be screened by the screening method of the present invention may be any antigen-binding molecules. For example, the above-described antigen-binding molecules can be screened. For example, it is possible to screen for antigen-binding molecules having a natural sequence or antigen-binding molecules having an amino acid sequence with a substitution.

Antigen-binding molecules to be screened by the screening method of the present invention may be prepared by any methods. It is possible to use, for example, pre-existing antibodies, pre-existing libraries (phage libraries, and such), and antibodies and libraries prepared from B cells of immunized animals or hybridomas prepared by immunizing animals, antibodies or libraries obtained by introducing amino acids capable of chelating calcium (for example, aspartic acid or glutamic acid) or non-natural amino acid mutations into such antibodies or libraries (libraries with high content of non-natural amino acids or amino acids capable of chelating calcium (for example, aspartic acid or glutamic acid), libraries introduced with non-natural amino acid mutations or mutations with amino acids capable of chelating calcium (for example, aspartic acid or glutamic acid) at specific sites, or such), or such.

An antigen-binding molecule having at least one function selected from:
(i) function of promoting antigen uptake into cells,
(ii) function of binding to an antigen two or more times,
(iii) function to promoting the reduction of plasma antigen concentration, and
(iv) function of excellence in plasma retention,
can be obtained by the screening methods of the present invention when administered to animals such as humans, mice, and monkeys. Thus, the screening methods of the present invention can be used as a screening method to obtain an antigen-binding molecule having at least one of the above-described functions.

Furthermore, such antigen-binding molecules obtained by the screening methods of the present invention are expected to be especially superior as pharmaceuticals, because the dose and frequency of administration in patients can be reduced, and as a result the total dosage can be

reduced. Thus, the screening methods of the present invention can be used as methods of screening for antigen-binding molecules for use as pharmaceutical compositions.

Methods for producing antigen-binding molecules

The present invention provides methods of producing an antigen-binding molecule that has a lower antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition. The present invention also provides methods of producing an antigen-binding molecule having at least one function selected from:

(i) function of promoting antigen uptake into cells,

(ii) function of binding to an antigen two or more times,

(iii) function of promoting the reduction of plasma antigen concentration, and

(iv) function of excellence in plasma retention.

Specifically, the present invention provides methods of producing an antigen-binding molecule, which comprise the steps of (a) to (e) below:

(a) determining the antigen-binding activity of an antigen-binding molecule under a low calcium concentration condition;

(b) determining the antigen-binding activity of the antigen-binding molecule under a high calcium concentration condition;

(c) selecting an antigen-binding molecule that has a lower antigen-binding activity under the low calcium concentration condition than under the high calcium concentration condition;

(d) obtaining a gene encoding the antigen-binding molecule selected in step (c); and

(e) producing the antigen-binding molecule using the gene obtained in step (d).

The present invention also provides methods of producing an antigen-binding molecule, which comprise the steps of (a) to (e) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a high calcium concentration condition;

(b) placing the antigen-binding molecule bound to the antigen in step (a) under a low calcium concentration condition;

(c) obtaining an antigen-binding molecule that dissociates in step (b);

(d) obtaining a gene encoding the antigen-binding molecule obtained in step (c); and

(e) producing the antigen-binding molecule using the gene isolated in step (d).

Steps (a) to (d) may be repeated two or more times. Thus, the present invention provides methods that further comprise the step of repeating steps (a) to (d) two or more times in the above-described methods. The number of times steps (a) to (d) are repeated is not particularly limited, and it is generally ten or less.

Furthermore, the present invention provides methods of producing an antigen-binding

molecule, which comprise the steps of (a) to (f) below:
(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a low calcium concentration condition;
(b) selecting an antigen-binding molecule that does not bind to the antigen in step (a);
(c) contacting the antigen with the antigen-binding molecule selected in step (b) under a high calcium concentration condition;
(d) obtaining an antigen-binding molecule that binds to the antigen in step (c);
(e) obtaining a gene encoding the antigen-binding molecule obtained in step (d); and
(f) producing the antigen-binding molecule using the gene obtained in step (e).

Steps (a) to (e) may be repeated two or more times. Thus, the present invention provides methods that further comprise the step of repeating steps (a) to (e) two or more times in the above-described methods. The number of times steps (a) to (e) are repeated is not particularly limited, and it is generally ten or less.

The present invention also provides methods of producing an antigen-binding molecule, which comprise the steps of (a) to (e) below:
(a) contacting an antigen-binding molecule or a library of antigen-binding molecules with an antigen-immobilized column under a high calcium concentration condition;
(b) eluting an antigen-binding molecule bound to the column in step (a) from the column under a low calcium concentration condition;
(c) obtaining the antigen-binding molecule eluted in step (b);
(d) obtaining a gene encoding the antigen-binding molecule obtained in step (c); and
(e) producing the antigen-binding molecule using the gene obtained in step (e).

Steps (a) to (d) may be repeated two or more times. Thus, the present invention provides methods that further comprise the step of repeating steps (a) to (d) two or more times in the above-described methods. The number of times steps (a) to (d) are repeated is not particularly limited, and it is generally ten or less.

The present invention also provides methods of producing an antigen-binding molecule, which comprise the steps of (a) to (f) below:
(a) allowing an antigen-binding molecule or a library of antigen-binding molecules to pass through an antigen-immobilized column under a low calcium concentration condition;
(b) collecting an antigen-binding molecule eluted without binding to the column in step (a);
(c) allowing the antigen-binding molecule collected in (b) to bind to the antigen under a high calcium concentration condition;
(d) obtaining an antigen-binding molecule that binds to the antigen in step (c);
(e) obtaining a gene encoding the antigen-binding molecule obtained in step (d); and
(f) producing an antigen-binding molecule using the gene obtained in step (e).

Steps (a) to (e) may be repeated two or more times. Thus, the present invention provides methods that further comprise the step of repeating steps (a) to (e) two or more times in the above-described methods. The number of times steps (a) to (e) are repeated is not particularly limited, and it is generally ten or less.

The present invention also provides methods of producing an antigen-binding molecule, which comprise the steps of (a) to (f) below:

(a) contacting an antigen with an antigen-binding molecule or a library of antigen-binding molecules under a high calcium concentration condition;

(b) obtaining an antigen-binding molecule that binds to the antigen in step (a);

(c) placing the antigen-binding molecule obtained in step (b) under a low calcium concentration condition;

(d) obtaining an antigen-binding molecule that has lower antigen-binding activity in step (c) than an antigen-binding activity in step (b);

(e) obtaining a gene encoding the antigen-binding molecule obtained in step (d); and

(f) producing the antigen-binding molecule using the gene obtained in step (e).

Steps (a) to (e) may be repeated two or more times. Thus, the present invention provides methods that further comprise the step of repeating steps (a) to (e) two or more times in the above-described methods. The number of times steps (a) to (e) are repeated is not particularly limited, and it is generally ten or less.

Antigen-binding molecules used in production methods of the present invention may be prepared by any method, and include, for example, existing antibodies and libraries (phage libraries, etc.), antibodies and libraries that are prepared from hybridomas obtained by immunizing animals or from B cells of immunized animals, antibodies and libraries prepared by introducing amino acids capable of chelating calcium (for example, aspartic acid and glutamic acid) or non-natural amino acid mutations into libraries (libraries with increased content of amino acids capable of chelating calcium (for example, aspartic acid and glutamic acid) or non-natural amino acids, libraries introduced with amino acids capable of chelating calcium (for example, aspartic acid and glutamic acid) or non-natural amino acid mutations at specific sites, or such).

In the above-described production methods, the antigen-binding activity of an antigen-binding molecule under a low calcium concentration condition is not particularly limited, as long as it is an antigen-binding activity at an ionized calcium concentration of 0.1 μM to 30 μM. Preferably, the antigen-binding activity includes an antigen-binding activity at an ionized calcium concentration of 0.5 μM to 10 μM. More preferred ionized calcium concentrations include the ionized calcium concentration in the early endosome *in vivo*. Specifically, the antigen-binding activity includes antigen-binding activities at 1 μM to 5 μM. Meanwhile, the

antigen-binding activity of an antigen-binding molecule under a high calcium concentration condition is not particularly limited, as long as it is an antigen-binding activity at an ionized calcium concentration of 100 μM to 10 mM. Preferably, the antigen-binding activity includes antigen-binding activities at an ionized calcium concentration of 200 μM to 5 mM. More preferred ionized calcium concentrations include the ionized calcium concentration in plasma *in vivo*. Specifically, the antigen-binding activity includes antigen-binding activities at 0.5 mM to 2.5 mM.

The antigen-binding activity of an antigen-binding molecule can be determined by methods known to those skilled in the art. Appropriate conditions other than the ionized calcium concentration may be determined by those skilled in the art.

The step of selecting an antigen-binding molecule that has greater antigen-binding activity under a high calcium concentration condition than under a low calcium concentration condition is synonymous with the step of selecting an antigen-binding molecule that has greater antigen-binding activity under a low calcium concentration condition than under a high calcium concentration condition.

The difference between the antigen binding activity under a high calcium concentration condition and that under a low calcium concentration condition is not particularly limited, as long as the antigen-binding activity is greater under a high calcium concentration condition than under a low calcium concentration condition. The antigen-binding activity under a high calcium concentration condition is preferably twice or more, more preferably 10 times or more, and still more preferably 40 times or more of the antigen-binding activity under a low calcium concentration condition.

In the production methods described above, the binding of an antigen and an antigen-binding molecule may be carried out in any state, and the state is not particularly limited. For example, the binding of an antigen and an antigen-binding molecule may be carried out by contacting an antigen with an immobilized antigen-binding molecule, or by contacting an antigen-binding molecule with an immobilized antigen. Alternatively, the binding can be carried out by contacting an antigen with an antigen-binding molecule in a solution.

Furthermore, the production method of the present invention may be used for an above-described antigen-binding molecule having the human FcRn-binding activity at neutral pH, or may be combined with a method of conferring or increasing the human FcRn-binding activity at neutral pH. When the production method of the present invention is combined with a method of conferring or increasing the human FcRn-binding activity at neutral pH, the method may additionally comprise the step of altering amino acids in the antigen-binding molecule to confer or increase the human FcRn-binding activity under a neutral pH condition. Meanwhile, the preferred human FcRn-binding domain of an antigen-binding molecule having the human FcRn-

binding activity at neutral pH includes, for example, the above-described human FcRn-binding domains having the human FcRn-binding activity at neutral pH. Thus, the production methods of the present invention may additionally comprise the step of selecting in advance an antigen-binding molecule having a human-FcRn-binding domain with greater human FcRn-binding activity at neutral pH and/or altering amino acids in an antigen-binding molecule to confer or increase the human FcRn-binding activity at neutral pH.

Furthermore, the production method of the present invention may be used for an antigen-binding molecule having the above-described pH-dependent antigen-binding activity, or may be combined with a method of conferring pH-dependent antigen-binding activity (WO 2009/125825). When the production method of the present invention is combined with a method of conferring pH-dependent antigen-binding activity, the method may additionally comprise the step of selecting in advance an antigen-binding molecule that has a lower antigen-binding activity under an acidic pH condition than under a neutral pH condition, and/or altering amino acids in an antigen-binding molecule to reduce the antigen-binding activity under an acidic pH condition to be lower than that under a neutral pH condition.

Preferred antigen-binding molecules having a pH-dependent antigen-binding activity include, for example, antigen-binding molecules in which at least one amino acid of an antigen binding molecule is substituted with histidine or at least one histidine is inserted into an antigen-binding molecule. Thus, the production method of the present invention may additionally comprise the step of using an antigen-binding molecule in which at least one amino acid is substituted with histidine or at least one histidine is inserted as an antigen-binding molecule, or the step of substituting histidine for at least one amino acid or inserting at least one histidine into an antigen-binding molecule.

In the production method of the present invention, non-natural amino acids may be used instead of histidine. Thus, the present invention can be understood with non-natural amino acids in place of histidine described above.

The production methods of the present invention can produce antigen-binding molecules having at least one function selected from:

(i) function of promoting antigen uptake into cells,

(ii) function of binding to an antigen two or more times,

(iii) function of promoting the reduction of plasma antigen concentration, and

(iv) function of excellence in plasma retention,

when administered to animals such as humans, mice, and monkeys. Thus, the production method of the present invention may be used as a method of producing an antigen-binding molecule having at least one of the above-described functions.

Furthermore, such antigen binding molecules are expected to be especially superior as

pharmaceuticals, because the dose and frequency of administration in patients can be reduced and as a result the total dosage can be reduced. Thus, the production methods of the present invention can be used as methods for producing antigen-binding molecules for use as pharmaceutical compositions.

Genes obtained by the production methods of the present invention are typically carried by (inserted into) appropriate vectors, and then introduced into host cells. The vectors are not particularly limited as long as they stably retain the inserted nucleic acids. For example, when *E. coli* is used as the host, preferred cloning vectors include the pBluescript vector (Stratagene); however, various commercially available vectors may be used. When using vectors to produce the antigen-binding molecules of the present invention, expression vectors are particularly useful. The expression vectors are not particularly limited as long as the vectors express the antigen-binding molecules *in vitro*, in *E. coli*, in culture cells, or in the body of an organism. For example, the pBEST vector (Promega) is preferred for *in vitro* expression; the pET vector (Invitrogen) is preferred for *E. coli*; the pME18S-FL3 vector (GenBank Accession No. AB009864) is preferred for culture cells; and the pME18S vector (Mol Cell Biol. (1988) 8: 466-472) is preferred for bodies of organisms. DNAs of the present invention can be inserted into the vectors by conventional methods, for example, by ligation using restriction enzyme sites (Current protocols in Molecular Biology, edit. Ausubel et al., (1987) Publish. John Wiley & Sons, Section 11.4-11.11).

The above host cells are not particularly limited, and various host cells may be used depending on the purpose. Examples of cells for expressing the antigen-binding molecules include bacterial cells (such as those of Streptococcus, Staphylococcus, *E. coli*, Streptomyces, and *Bacillus subtilis*), eukaryotic cells (such as those of yeast and Aspergillus), insect cells (such as Drosophila S2 and Spodoptera SF9), animal cells (such as CHO, COS, HeLa, C127, 3T3, BHK, HEK293, and Bowes melanoma cells), and plant cells. Vectors can be introduced into a host cell by known methods, for example, calcium phosphate precipitation methods, electroporation methods (Current protocols in Molecular Biology edit. Ausubel et al. (1987) Publish. John Wiley & Sons, Section 9.1-9.9), lipofection methods, and microinjection methods.

The host cells can be cultured by known methods. For example, when using animal cells as a host, DMEM, MEM, RPMI1640, or IMDM may be used as the culture medium. They may be used with serum supplements such as FBS or fetal calf serum (FCS). The cells may be cultured in serum-free cultures. The preferred pH is about 6 to 8 during the course of culturing. Incubation is carried out typically at about 30 to 40°C for about 15 to 200 hours. Medium is exchanged, aerated, or agitated, as necessary.

Appropriate secretion signals may be incorporated to polypeptides of interest so that the antigen-binding molecules expressed in the host cell are secreted into the lumen of the

endoplasmic reticulum, periplasmic space, or extracellular environment. These signals may be endogenous to the antigen-binding molecules of interest or may be heterologous signals.

On the other hand, for example, production systems using animals or plants may be used as systems for producing polypeptides *in vivo*. A polynucleotide of interest is introduced into an animal or plant and the polypeptide is produced in the body of the animal or plant, and then collected. The "hosts" of the present invention include such animals and plants.

The production system using animals include those using mammals or insects. It is possible to use mammals such as goats, pigs, sheep, mice, and bovines (Vicki Glaser SPECTRUM Biotechnology Applications (1993)). The mammals may be transgenic animals.

For example, a polynucleotide encoding an antigen-binding molecule of the present invention is prepared as a fusion gene with a gene encoding a polypeptide specifically produced in milk, such as the goat β casein. Next, goat embryos are injected with polynucleotide fragments containing the fusion gene, and then transplanted to female goats. Desired antigen-binding molecules can be obtained from milk produced by the transgenic goats, which are born from the goats that received the embryos, or from their offspring. Hormones may be administered as appropriate to increase the volume of milk containing the antigen-binding molecule produced by the transgenic goats (Ebert et al., Bio/Technology (1994) 12: 699-702).

Insects such as silkworms may be used to produce the antigen-binding molecules of the present invention. When silkworms are used, baculoviruses carrying a polynucleotide encoding an antigen-binding molecule of interest can be used to infect silkworms, and the antigen-binding molecule of interest can be obtained from their body fluids.

Furthermore, when plants are used to produce the antigen-binding molecules of the present invention, for example, tobacco may be used. When tobacco is used, a polynucleotide encoding an antigen-binding molecule of interest is inserted into a plant expression vector, for example, pMON 530, and then the vector is introduced into bacteria, such as *Agrobacterium tumefaciens*. The bacteria are then allowed to infect tobacco such as *Nicotiana tabacum*, and the desired antigen-binding molecules can be collected from their leaves (Ma et al., Eur. J. Immunol. (1994) 24: 131-138). Alternatively, it is possible to infect duckweed (*Lemna minor*) with similar bacteria. After cloning, the desired antigen-binding molecules can be obtained from the duckweed cells (Cox KM et al., Nat. Biotechnol. 2006 Dec; 24(12): 1591-1597).

The thus obtained antigen-binding molecules may be isolated from the inside or outside (such as the medium and milk) of host cells, and purified as substantially pure and homogenous antigen-binding molecules. The methods for isolating and purifying antigen-binding molecules are not particularly limited, and isolation and purification methods usually used for polypeptide purification can be used. Antigen-binding molecules may be isolated and purified by appropriately selecting and combining, for example, chromatographic columns, filtration,

ultrafiltration, salting out, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectric focusing, dialysis, and recrystallization.

Chromatography includes, for example, affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel filtration, reverse-phase chromatography, and adsorption chromatography (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak *et al.*, (1996) Cold Spring Harbor Laboratory Press). Such chromatographic methods can be conducted using liquid phase chromatography such as HPLC and FPLC. Columns used for affinity chromatography include, protein A columns and protein G columns. Columns using protein A include, for example, Hyper D, POROS, and Sepharose F. F. (Pharmacia).

If needed, an antigen-binding molecule can be modified arbitrarily, and peptides can be partially deleted by allowing an appropriate protein modification enzyme to act before or after purification of the antigen-binding molecule. Such protein modification enzymes include, for example, trypsin, chymotrypsin, lysyl endopeptidases, protein kinases, and glucosidases.

<Pharmaceutical compositions>

The present invention also relates to pharmaceutical compositions that include antigen-binding molecules of the present invention, antigen-binding molecules isolated by the screening methods of the present invention, or antigen-binding molecules produced by the production methods of the present invention. Antigen-binding molecules of the present invention, antigen-binding molecules isolated by the screening method of the present invention, or antigen-binding molecules produced by the production method of the present invention are antigen-binding molecules having at least one function selected from:

(i) function of promoting antigen uptake into cells,

(ii) function of binding to an antigen two or more times,

(iii) function of promoting the reduction of plasma antigen concentration, and

(iv) function of excellence in plasma retention,

are useful as pharmaceutical compositions, because it is expected that the administration frequency can be reduced. Furthermore, the pharmaceutical composition of the present invention may comprise a pharmaceutically acceptable carrier.

In the present invention, pharmaceutical compositions generally refer to agents for treating or preventing, or testing and diagnosing diseases.

The pharmaceutical compositions of the present invention can be formulated by methods known to those skilled in the art. For example, they can be used parenterally, in the form of injections of sterile solutions or suspensions including water or other pharmaceutically

acceptable liquid. For example, such compositions may be formulated by mixing in the form of unit dose required in the generally approved medicine manufacturing practice, by appropriately combining with pharmacologically acceptable carriers or media, specifically with sterile water, physiological saline, vegetable oil, emulsifier, suspension, surfactant, stabilizer, flavoring agent, excipient, vehicle, preservative, binder, or such. In such formulations, the amount of active ingredient is adjusted to obtain an appropriate amount in a pre-determined range.

Sterile compositions for injection can be formulated using vehicles such as distilled water for injection, according to standard formulation practice.

Aqueous solutions for injection include, for example, physiological saline and isotonic solutions containing dextrose or other adjuvants (for example, D-sorbitol, D-mannose, D-mannitol, and sodium chloride). It is also possible to use in combination appropriate solubilizers, for example, alcohols (ethanol and such), polyalcohols (propylene glycol, polyethylene glycol, and such), non-ionic surfactants (polysorbate 80(TM), HCO-50, and such).

Oils include sesame oil and soybean oils. Benzyl benzoate and/or benzyl alcohol can be used in combination as solubilizers. It is also possible to combine buffers (for example, phosphate buffer and sodium acetate buffer), soothing agents (for example, procaine hydrochloride), stabilizers (for example, benzyl alcohol and phenol), and/or antioxidants. Appropriate ampules are filled with the prepared injections.

The pharmaceutical compositions of the present invention are preferably administered parenterally. For example, the compositions may be in the dosage form for injections, transnasal administration, transpulmonary administration, or transdermal administration. For example, they can be administered systemically or locally by intravenous injection, intramuscular injection, intraperitoneal injection, subcutaneous injection, or such.

Administration methods can be appropriately selected in consideration of the patient's age and symptoms. The dose of a pharmaceutical composition containing an antigen-binding molecule may be, for example, from 0.0001 to 1,000 mg/kg for each administration. Alternatively, the dose may be, for example, from 0.001 to 100,000 mg per patient. However, the present invention is not limited by the numeric values described above. The doses and administration methods vary depending on the patient's weight, age, symptoms, and such. Those skilled in the art can set appropriate doses and administration methods in consideration of the factors described above.

Furthermore, the pharmaceutical composition of the present invention may be a pharmaceutical composition used to promote antigen uptake into cells or reduction of antigen concentration in plasma.

The present invention also relates to methods of promoting antigen uptake into cells by an antigen-binding molecule and methods of promoting the reduction of antigen concentration in

plasma by administering the antigen-binding molecule of the present invention or antigen-binding molecule produced by the production method of the present invention. The antigen-binding molecule may be administered *in vivo* or *in vitro*. The subject to be administered includes, for example, nonhuman animals (mice, monkeys, etc.) and humans.

The present invention also relates to methods of increasing the number of times of antigen binding by one antigen-binding molecule and methods of improving the plasma retention of an antigen-binding molecule by using an antigen-binding molecule of the present invention or an antigen-binding molecule produced by the production method of the present invention.

Amino acids contained in the amino acid sequences of the present invention may be post-translationally modified (for example, the modification of an N-terminal glutamine into a pyroglutamic acid by pyroglutamylation is well-known to those skilled in the art). Naturally, such post-translationally modified amino acids are included in the amino acid sequences in the present invention.

Furthermore, the present invention provides kits for use in the methods of the present invention, which comprise at least an antigen-binding molecule of the present invention. In addition to the above, pharmaceutically acceptable carriers, media, instruction manuals describing the using method, and such may be packaged into the kits.

The present invention also relates to agents for promoting antigen uptake into cells by antigen-binding molecules, agents for promoting the reduction of plasma antigen concentration, agents for increasing the number of times of antigen binding by one antigen-binding molecule, and agents for improving plasma retention of antigen-binding molecules, all of which comprise as an active ingredient an antigen-binding molecule of the present invention or an antigen-binding molecule produced by production methods of the present invention.

The present invention also relates to the use of antigen-binding molecules of the present invention or antigen-binding molecules produced by production methods of the present invention in producing agents for promoting antigen uptake into cells by antigen-binding molecules, agents for promoting the reduction of plasma antigen concentration, agents for increasing the number of times of antigen binding by one antigen-binding molecule, or agents for improving plasma retention of antigen-binding molecules.

The present invention also relates to antigen-binding molecules of the present invention or antigen-binding molecules produced by production methods of the present invention for use in methods for promoting antigen uptake into cells by the antigen-binding molecules, agents for promoting the reduction of plasma antigen concentration, methods for increasing the number of times of antigen binding by one antigen-binding molecule, and methods for improving plasma retention of antigen-binding molecules.

All prior art documents cited in the specification are incorporated herein by reference.

[Examples]

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

[Example 1] The concept of antigen elimination-accelerating effect of calcium-dependent antigen-binding antibodies

(1-1) Effect of pH-dependent antigen-binding antibodies to accelerate antigen elimination

H54/L28-IgG1 described in WO 2009/125825 is a humanized anti-IL-6 receptor antibody. Fv4-IgG1 is a humanized anti-IL-6 receptor antibody that results from conferring H54/L28-IgG1 with the property to bind to soluble human IL-6 receptor in a pH-dependent manner (which binds under neutral condition but is dissociated under acidic condition). The *in vivo* test described in WO 2009/125825 using mice demonstrated that the elimination of soluble human IL-6 receptor could be greatly accelerated in a group administered with a mixture of Fv4-IgG1 and soluble human IL-6 receptor as antigen as compared to a group administered with a mixture of H54/L28-IgG1 and soluble human IL-6 receptor as antigen.

Soluble human IL-6 receptor bound to a general antibody that binds to soluble human IL-6 receptor is recycled to the plasma along with the antibody *via* FcRn. Meanwhile, an antibody that binds to soluble human IL-6 receptor in a pH-dependent manner dissociates from the soluble human IL-6 receptor that has been bound to the antibody under acidic conditions in the endosome. The dissociated soluble human IL-6 receptor is degraded in the lysosome. This can greatly accelerate the elimination of soluble human IL-6 receptor. Then, the antibody that binds to soluble human IL-6 receptor in a pH-dependent manner is recycled to the plasma *via* FcRn. The recycled antibody can bind to a soluble human IL-6 receptor again. By repeating this cycle, a single antibody molecule can repeatedly bind to soluble human IL-6 receptors multiple times (Fig. 1).

Meanwhile, as described in WO 2009/125825, after binding to membrane-type human IL-6 receptor, a general humanized anti-IL-6 receptor antibody is internalized in a complex of humanized anti-IL-6 receptor antibody and membrane-type human IL-6 receptor and then degraded in the lysosome. In contrast, a humanized anti-IL-6 receptor antibody that binds to IL-6 receptor in a pH-dependent manner is recycled to plasma *via* dissociation from the membrane-type human IL-6 receptor under the acidic condition in the endosome after internalization in a complex with membrane-type human IL-6 receptor. The recycled antibody can bind to membrane-type human IL-6 receptor again. By repeating this cycle, a single antibody molecule can repeatedly bind to membrane-type human IL-6 receptor multiple times (Fig. 2).

(1-2) pH and calcium concentrations in plasma and endosome

In the mechanism of a pH-dependent binding antibody shown in Figs. 1 and 2, it is important that the antibody strongly binds to an antigen in plasma and dissociates from the antigen in the endosome based on the environmental difference between plasma and endosome, i.e., pH difference (pH 7.4 in plasma; pH 6.0 in endosome). The degree of environmental difference between plasma and endosome is important for differentiating the antigen-binding ability of a pH-dependent binding antibody in plasma and endosome. A pH difference is due to a difference in the hydrogen ion concentration. Specifically, the hydrogen ion concentration in plasma (pH 7.4) is about 40 nM, while the concentration in the endosome (pH 6.0) is about 1,000 nM. The factor (hydrogen ion) concentration differs by about 25 times between plasma and endosome.

The present inventors conceived that, in order to achieve the mechanism illustrated in Figs. 1 and 2 easily or to enhance the mechanism, it would be beneficial to use an antibody that depends on a factor that has a greater concentration difference between plasma and endosome than the difference of hydrogen ion concentration between the two. Thus, the inventors searched for a factor whose concentration is considerably different between plasma and endosome. As a result, calcium was identified. The ionized calcium concentration is about 1.1 to 1.3 mM in plasma and about 3 μM in the endosome. The factor (calcium) concentration differs by about 400 times between the two. Thus, the ratio was found to be greater than the difference in hydrogen ion concentration (25 times). Specifically, the mechanism illustrated in Figs. 1 and 2 was expected to be achieved or enhanced more readily by using an ionized calcium concentration-dependent binding antibody, which binds to an antigen under a high calcium concentration condition (1.1 to 1.3 mM) but dissociates from the antigen under a low calcium concentration condition (3 μM).

Furthermore, in WO 2009/125825, pH-dependent binding antibodies whose properties change between pH 7.4 and 6.0 were produced by introducing histidine. Histidine is electrically neutral under the neutral condition in plasma but is positively charged under the acidic condition in the endosome. The pH dependency can be conferred to antigen-antibody interaction by utilizing the change in the electric charge of histidine. Meanwhile, as shown in Fig. 3, when histidine is used, in order to bind to an antigen in plasma and to dissociate from the antigen in the endosome, histidine residues in the antibody need to interact with antigen's positively charged amino acids or amino acids that potentially serve as a donor for hydrogen bonding. Therefore, an antigen epitope, to which a pH-dependent binding antibody binds to exert a target effect, has to contain positively charged amino acids or amino acids that potentially serve as a donor for hydrogen bonding.

On the other hand, as shown in Fig. 4, a calcium-dependent binding antibody is assumed to bind to an antigen *via* calcium ion. In this case, the antigen epitope contains negatively charged amino acids or amino acids that potentially serve as an acceptor for hydrogen bonding, which are capable of chelating calcium ion. Thus, such antibodies can target epitopes that are not targeted by pH-dependent binding antibodies produced by introducing histidine. Furthermore, as shown in Fig. 5, it is expected that epitopes that have a wide variety of properties can be targeted by using antibodies with both calcium dependency and pH dependency.

[Example 2] Isolation of Ca-dependent binding antibodies from human antibody library using phage-display technique

(2-1) Preparation of phage-display library of naive human antibodies

Several human antibody phage-display libraries that present Fab domains comprising a human antibody sequence were constructed using as a template polyA-RNA prepared from human PBMC, human polyA RNA available on the market, or the like, according to Methods Mol Biol. 2002, 178: 87-100.

(2-2) Isolation of Ca-dependent binding antibody fragments from libraries by bead panning

The first selection from constructed human antibody phage-display libraries was achieved by enriching antibody fragments having antibody-binding ability or by enriching using the Ca-dependent binding ability as an indicator. Antibody fragments with a Ca-dependent binding ability were enriched by eluting phages *via* EDTA chelation of Ca ion after antibody fragments were bound to an antigen in the presence of Ca ion. Biotinylated human IL-6 receptor was used as the antigen.

Phages were produced with *E. coli* carrying phage-display phagemids constructed in the manner described above. The resulting culture medium was precipitated using 2.5 M NaCl/10% PEG. Then, the precipitate was diluted with TBS to prepare a phage library solution. BSA and $CaCl_2$ were added to the phage library solution so that the final concentrations of BSA and ionized calcium were 4% and 1.2 mM, respectively. Panning was carried out according to a conventional panning method using antigen-immobilized magnetic beads (J Immunol Methods. 2008 Mar 20, 332(1-2): 2-9; J Immunol Methods. 2001 Jan 1, 247(1-2): 191-203; Biotechnol Prog. 2002 Mar-Apr, 18(2): 212-20; Mol Cell Proteomics. 2003 Feb, 2(2): 61-9). The magnetic beads used were NeutrAvidin coated beads (Sera-Mag SpeedBeads NeutrAvidin-coated) and Streptavidin coated beads (Dynabeads M-280 Streptavidin).

Specifically, 250 pmol of the biotinylated antigen was added to the prepared phage library solution, and contacted with the antigen at room temperature for 60 minutes. BSA-

blocked magnetic beads were added and incubated for binding at room temperature for 15 minutes. The beads were washed once with 1 mL of 1.2 mM $CaCl_2$/TBS (TBS containing 1.2 mM $CaCl_2$). Then, the phages were harvested by elution using a standard method when enriching antibody fragments having binding ability, or by suspending the beads in 2 mM EDTA/TBS (TBS containing 2% EDTA) to enrich antibody fragments having Ca-dependent binding ability. *E. coli* was infected by adding 10 mL of the *E. coli* strain TG1 during the logarithmic growth phase (OD600 0.4-0.5) to the prepared phage suspension, and culturing at 37°C for one hour with gentle stirring. The infected *E. coli* was plated onto plates (225 mm x 225 mm). Again, the culture was started with this *E. coli* to cultivate the phages.

In the second and subsequent panning, the enrichment was achieved using Ca-dependent binding ability as an indicator. Specifically, 40 pmol of the biotinylated antigen was added to the prepared phage library solution. The phages were contacted with the antigen at room temperature for 60 minutes. BSA-blocked magnetic beads were added to the suspension and incubated for binding at room temperature for 15 minutes. The beads were washed once each with 1 mL of 1.2 mM $CaCl_2$/TBST (TBS containing 1.2 mM $CaCl_2$ and 0.1% Tween-20) and 1.2 mM $CaCl_2$/TBS. Then, 0.1 mL of 2 mM EDTA/TBS (TBS containing 2% EDTA) was added to suspend the beads at room temperature, and immediately after suspension, the beads were removed using Magnet Stand to collect the phage suspension. The resulting phage suspension was added to 10 mL of the *E. coli* stain TG1 during the logarithmic growth phase (OD600 0.4-0.5) to infect the *E. coli* which was then cultured at 37°C for one hour with gentle stirring. The infected *E. coli* was plated onto plates (225 mm x 225 mm). Again, the culture was started with this *E. coli*, and the phages were cultivated in the manner as described above. Panning was repeated twice.

(2-3) Assessment by phage ELISA

From *E. coli* single colonies obtained by the method described above, phage-containing culture supernatants were prepared according to Methods Mol Biol. 2002, 178: 133-145.

BSA and $CaCl_2$ were added to the phage-containing culture supernatants so that the final concentrations of BSA and calcium were 4% and 1.2 mM, respectively. The supernatants were subjected to ELISA. StreptaWell 96 microtiter plates (Roche) were coated using 100 µL of PBS containing the biotinylated antigen. After washing with PBST (PBS containing 0.1% Tween20) to remove the antigen, the plates were blocked with 250 µL of 4% BSA/TBS for one hour or more. 4% BSA-TBS was removed, and then the prepared culture supernatants were added to the plates. The plates were allowed to stand at 37°C for one hour to achieve the binding of phage-display antibody. Following wash with 1.2 mM $CaCl_2$/TBST (TBS containing 1.2 mM $CaCl_2$ and 0.1% Tween20), 1.2 mM $CaCl_2$/TBS or 1 mM EDTA/TBS was added to the plates.

The plates were allowed to stand at 37°C for 30 minutes of incubation. After washing with 1.2 mM $CaCl_2$/TBST, the plates were incubated for one hour with an HRP-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) diluted with TBS containing 4% BSA and 1.2 mM ionized calcium. After washing with 1.2 mM $CaCl_2$/TBST, detection was carried out with the TMB single solution (ZYMED). Absorbance at 450 nm was determined after the reaction was terminated by adding sulfuric acid. Antibody fragments judged to have a Ca-dependent binding ability were analyzed for their nucleotide sequences using specific primers.

(2-4) Antibody expression and purification

Clones judged to have a Ca-dependent binding ability by phage ELISA were introduced into animal cell expression plasmids. Antibodies were expressed using the following method. Cells of human fetal kidney-derived line FreeStyle 293-F (Invitrogen) were suspended in the FreeStyle 293 Expression Medium (Invitrogen), and 3-ml aliquots were plated to each well of 6-well plates at a cell density of 1.33×10^6 cells/mL. The prepared plasmids were introduced into the cells by a lipofection method. The cells were cultured in a CO_2 incubator (37°C, 8% CO_2, 90 rpm) for four days. From the obtained culture supernatants, antibodies were purified using rProtein A Sepharose™ Fast Flow (Amersham Biosciences) by a method known to those skilled in the art. The concentrations of purified antibodies were determined by measuring absorbance at 280 nm using a spectrophotometer. The antibody concentrations were calculated from the determined values based on the extinction coefficient determined by PACE method (Protein Science 1995; 4: 2411-2423).

[Example 3] Assessment of the prepared antibodies for their Ca-dependent binding activity to human IL-6 receptor

Antibodies 6RL#9-IgG1 (heavy chain SEQ ID NO: 1; light chain SEQ ID NO: 2), 6RK#12-IgG1 (heavy chain SEQ ID NO: 66; light chain SEQ ID NO: 67), and FH4-IgG1 (heavy chain SEQ ID NO: 3; light chain SEQ ID NO: 4) prepared in Example 2 were assessed for their binding activity to human interleukin 6 receptor (hIL6R) at pH 7.4 using Biacore T100 (GE Healthcare). The assay was carried out using as a running buffer 0.05% Surfactant P20, 10 mmol/l ACES, 150 mmol/l NaCl (pH 7.4 or 6.0) containing 3 μM or 2 mM $CaCl_2$.

After immobilizing an adequate amount of recombinant Protein A (Thermo Scientific) onto Sensor chip CM4 (GE Healthcare) by an amino coupling method, antibodies were allowed to bind onto the sensor chip. An appropriate concentration of hIL-6R was injected as an analyte to interact with antibodies on the sensor chip. Then, 10 mmol/l glycine-HCl (pH 1.5) was injected to regenerate the sensor chip. Measurements were carried out at 37°C. Sensorgrams obtained by the measurements are show in in Fig. 6. The result demonstrated that all of

antibodies 6RL#9-IgG1, 6RK#12-IgG1, and FH4-IgG1 bound to hIL6R more weakly under the condition of 3 μM Ca^{2+} concentration of than under the condition of 2 mM Ca^{2+} concentration.

Of these antibodies, as antibodies exhibiting Ca dependency, 6RL#9-IgG1 (heavy chain SEQ ID NO: 1; light chain SEQ ID NO: 2) and FH4-IgG1 (heavy chain SEQ ID NO: 3; light chain SEQ ID NO: 4) were further analyzed kinetically. H54/L28-IgG1 (heavy chain SEQ ID NO: 5; light chain SEQ ID NO: 6) described in WO 2009/125825 was used as an antibody exhibiting no Ca dependency. The high and low calcium ion concentration conditions used were 2 mM and 3 μM, respectively. Human IL-6 receptor (IL-6R) was used as an antigen. An appropriate amount of protein A (Invitrogen) was immobilized onto Sensor chip CM4 (GE Healthcare) by the amine coupling method and antibodies of interest were captured on the chip. The two types of running buffers used were: [10 mmol/L ACES, 150 mmol/L NaCl, 0.05% (w/v) Tween20, 2 mmol/L $CaCl_2$ (pH 7.4)] or [10 mmol/L ACES, 150 mmol/L NaCl, 0.05% (w/v) Tween20, 3 μmol/L $CaCl_2$ (pH 7.4)]. All measurements were carried out at 37°C. Each buffer was also used to dilute IL-6R.

H54L28-IgG1 was assayed by injecting each running buffer as a blank and the diluted IL-6R solution at a flow rate of 20 μl/min for three minutes. Thus, IL-6R was allowed to interact with the antibody captured on the sensor chip. Then, the running buffer was injected at a flow rate of 20 μl/min for ten minutes to observe the dissociation of IL-6R. Next, 10 mmol/L glycine-HCl (pH 1.5) was injected at a flow rate of 30 μl/min for 30 seconds to regenerate the sensor chip. Association rate constant ka (1/Ms) and dissociation rate constant kd (1/s), which are kinetic parameters, were calculated from the sensorgram obtained by the measurement. Based on the values, the dissociation constant K_D (M) between each antibody and human IL-6 receptor was calculated. Each parameter was calculated using the Biacore T100 Evaluation Software (GE Healthcare).

FH4-IgG1 and 6RL#9-IgG1 were assayed by injecting each running buffer as a blank and the diluted IL-6R solution at a flow rate of 5 μl/min for 15 minutes. Thus, IL-6R was allowed to interact with the antibody captured on the sensor chip. Then, 10 mmol/L glycine-HCl (pH 1.5) was injected at a flow rate of 30 μl/min for 30 seconds to regenerate the sensor chip. Based on the steady state affinity model, the dissociation constant K_D (M) was calculated from the sensorgram obtained by the measurement. Each parameter was calculated using the Biacore T100 Evaluation Software (GE Healthcare).

The dissociation constants K_D between IL-6R and each antibody in the presence of 2 mM $CaCl_2$, which was determined by the above-described methods, are shown in Table 7. H54/L28-IgG1 did not show any difference in the level of IL-6R binding due to the Ca concentration difference. Meanwhile, FH4-IgG1 and 6RL#9-IgG1 exhibited a significant impairment of binding at the low Ca concentration condition (Figs. 7, 8, and 9).

[Table 7]

	H54/L28-IgG1	FH4-IgG1	6RL#9-IgG1
K_D (M)	1.9E-9	5.9E-7	2.6E-7

In the case of H54/L28-IgG1, K_D at a Ca concentration of 3 μM can be calculated by similar methods used for determining K_D at a Ca concentration of 2 mM. In the case of FH4-IgG1 and 6RL#9-IgG1, on the other hand, it is difficult to calculate K_D at a Ca concentration of 3 μM by similar methods described above, because the binding to IL-6R was almost undetectable at 3 μM Ca concentration. However, the K_D can be predicted by using formula 1 shown below (Biacore T100 Software Handbook, BR-1006-48, AE 01/2007).

[Formula 1]

$$R_{eq}=C \bullet R_{max} / (K_D+C)+RI$$

Each symbol in formula 1 shown above is defined below.

R_{eq} (RU): steady state binding levels

R_{max} (RU): analyte binding capacity of the surface

RI (RU): bulk refractive index contribution in the sample

C (M): analyte concentration

K_D (M): equilibrium dissociation constant

The dissociation constant K_D between IL-6R and each antibody at a Ca concentration of 3 μmol/L, which can be predicted by using formula 1 above, is shown as an approximate estimate in Table 8.

[Table 8]

	H54L28-IgG1	FH4-IgG1	6RL#9-IgG1
R_{eq} (RU)		5	10
R_{max} (RU)		39	72
RI (RU)		0	0
C (M)		5E-06	5E-06
K_D (M)	2.2E-9	3.4E-05	3.1E-05

In Table 8 shown above, the R_{eq}, R_{max}, RI, and C values are estimated based on the assay result.

Based on the findings described above, it was predicted that the K_D between IL-6R and FH4-IgG1 or 6RL#9-IgG1 was increased by about 60 or 120 times (the affinity was reduced by 60 or 120 times or more) when the concentration of $CaCl_2$ was altered from 2 mM to 3μM. Table 9 summarizes the K_D values at $CaCl_2$ concentrations of 2 mM and 3 μM and the Ca dependency for the three types of antibodies H54/L28-IgG1, FH4-IgG1, and 6RL#9-IgG1.

[Table 9]

	H54/L28-IgG1	FH4-IgG1	6RL#9-IgG1
K_D (M) (2mM $CaCl_2$)	1.9E-9	5.9E-7	2.6E-7
K_D (M) (3μM $CaCl_2$)	2.2E-9	3.4E-5 OR HIGHER	3.1E-5 OR HIGHER
Ca DEPENDENCY	ABOUT THE SAME	ABOUT 60 FOLD OR MORE	ABOUT 120 FOLD OR MORE

[Example 4] Assessment of the obtained antibodies for their calcium ion binding

Next, antibodies were tested for their calcium ion binding by differential scanning calorimetry (DSC) (MicroCal VP-Capillary DSC; MicroCal) to assess the midpoint temperature of thermal denaturation (Tm value). The midpoint temperature of thermal denaturation (Tm value) serves as an indicator for stability. When a protein is stabilized by calcium ion binding, the midpoint temperature of thermal denaturation (Tm value) is elevated as compared to that when the protein is not bound to calcium ion (J Bio Chem. 2008 Sep 12; Vol.283; No. 37: pp 25140-25149). Based on this principle, antibodies were assessed for their calcium ion binding. Purified antibodies were dialyzed (EasySEP, TOMY) against a solution of [20 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$ (pH 7.4)] or [20 mM Tris-HCl, 150 mM NaCl, 3 μM $CaCl_2$ (pH 7.4)]. The protein solutions were adjusted to 0.1 mg/ml using the same dialysis buffer as used in dialyzing the protein solution. DSC measurement was carried out at a heating rate of 240°C/hr from 20 to 115°C. Based on the obtained DSC denaturation curves, the midpoint temperature of thermal denaturation (Tm value) was calculated for the Fab domain of each antibody. The values are shown in Table 10.

[Table 10]

VARIABLE REGION SEQUENCE	CALCIUM ION CONCENTRATION		ΔTm[°C]
	3μM	2mM	2mM - 3μM
H54/L28	92.87	92.87	0.00
FH4	74.71	78.97	4.26
6RL#9	77.77	78.98	1.21

The result shown in Table 10 demonstrates that for FH4 and 6RL#9, which exhibit calcium-dependent binding ability, the Tm values of their Fab vary depending on the calcium concentration, while the Tm value does not change in H54/L28, which does not exhibit calcium-dependent binding ability. The observed changes in the Tm values of Fab in FH4 and 6RL#9 suggest that the Fab domains of the antibodies were stabilized by calcium ion binding to the antibodies. This implies that calcium ion binds to FH4 and 6RL#9 whereas calcium ion does not bind to H54/L28.

[Example 5] Assessment of Ca-dependent binding antibodies for their effect on antigen retention in plasma using normal mice

(5-1) *In vivo* test using normal mice

Normal mice (57BL/6J mouse; Charles River Japan) were administered with hsIL-6R (soluble human IL-6 receptor: prepared as described in REFERENCE EXAMPLE 1) alone or in combination with an anti-human IL-6 receptor antibody, and then assessed for the *in vivo* dynamics of hsIL-6R and the anti-human IL-6 receptor antibody. An hsIL-6R solution (5 μg/ml) or a mixed solution of hsIL-6R and an anti-human IL-6 receptor antibody was administered at 10 ml/kg once into the caudal vein. The anti-human IL-6 receptor antibodies used were H54/L28-IgG1, 6RL#9-IgG1, and FH4-IgG1 described above.

The concentration of hsIL-6R was 5 μg/ml in all the mixed solutions. Meanwhile, the anti-human IL-6 receptor antibody concentration differs with each antibody. The concentration of H54/L28-IgG1 was 0.1 mg/mL, while those of 6RL#9-IgG1 and FH4-IgG1 were 10 mg/mL. The anti-human IL-6 receptor antibody is present in excess over hsIL-6R, and therefore almost every hsIL-6R is assumed to be bound by the antibody. Blood was collected 15 minutes, 7 hours, 1 day, 2 days, 4 days, 7 days, 14 days, 21 days, and 28 days after administration. The collected blood was immediately centrifuged at 4°C and 12,000 rpm for 15 minutes to separate the plasma. The separated plasma was stored in a freezer at -20°C or below until measurement.

(5-2) ELISA determination of the anti-human IL-6 receptor antibody concentration in normal mice plasma

The anti-human IL-6 receptor antibody concentration in mouse plasma was determined by ELISA. First, Anti-Human IgG (γ-chain specific) F(ab')2 Fragment of Antibody (SIGMA) was dispensed onto Nunc-Immuno Plates, MaxiSorp (Nalge nunc International) and allowed to stand overnight at 4°C to prepare Anti-Human IgG-immobilized plates. Calibration curve samples having plasma concentrations of 0.64, 0.32, 0.16, 0.08, 0.04, 0.02, and 0.01 μg/mL, and mouse plasma assay samples diluted 100-fold or more were prepared and aliquoted into the Anti-

Human IgG-immobilized plates. The plates were incubated at 25°C for one hour, followed by incubation with biotinylated anti-human IL-6R antibody (R&D) at 25°C for one hour. Then, Streptavidin-PolyHRP80 (Stereospecific Detection Technologies) was reacted at 25°C for 0.5 hour. Color development was carried out using TMB One Component HRP Microwell Substrate (BioFX Laboratories) as a substrate. After stopping the reaction with 1N Sulfuric acid (Showa Chemical), absorbance at 450 nm was measured on a microplate reader. The plasma concentrations in the mice were calculated from the absorbance of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices). Time courses for the plasma concentrations of antibodies H54/L28-IgG1, 6RL#9-IgG1, and FH4-IgG1 in normal mice after intravenous administration determined by this method are shown in Fig. 10.

(5-3) Measurement of plasma hsIL-6R concentration by electrochemiluminescence method

The hsIL-6R concentration in mouse plasma was measured by the electrochemiluminescence method. hsIL-6R calibration curve samples adjusted to concentrations of 2,000, 1,000, 500, 250, 125, 62.5, or 31.25 pg/mL and mouse plasma assay samples diluted 50-fold or more were prepared. The samples were mixed with a solution of monoclonal anti-human IL-6R antibody (R&D) ruthenium-labeled with SULFO-TAG NHS Ester (Meso Scale Discovery), Biotinylated Anti-human IL-6R Antibody (R&D), and tocilizumab (heavy chain SEQ ID NO: 13; light chain SEQ ID NO: 14), and then allowed to react overnight at 4°C. The assay buffer used for the reaction contains 10 mM EDTA for the purpose of reducing the free Ca concentration in the samples so that almost every hsIL-6R is dissociated from 6RL#9-IgG1 or FH4-IgG1 in the samples and binds to tocilizumab added. Then, the mixtures were aliquoted into the MA400 PR Streptavidin Plate (Meso Scale Discovery). After another hour of reaction at 25°C, the plate was washed. Immediately after Read Buffer T(x4) (Meso Scale Discovery) was aliquoted into the plate, measurement was carried out using the SECTOR PR 400 reader (Meso Scale Discovery). The hSIL-6R concentration was calculated based on the response in the calibration curve using the analytical software, SOFTmax PRO (Molecular Devices). Time courses of the plasma hsIL-6R concentration in normal mice after intravenous administration determined by the above-described method are shown in Fig. 11.

The findings described above demonstrated that hsIL-6R administered alone was eliminated very rapidly. Meanwhile, the elimination of hsIL-6R was considerably retarded when hsIL-6R was simultaneously administered with a general antibody H54/L28-IgG1 which does not exhibit Ca-dependent hsIL-6R binding. Meanwhile, the elimination of hsIL-6R was significantly accelerated when hsIL-6R was simultaneously administered with 6RL#9-IgG1 or FH4-IgG1, which has 100 times or higher hsIL-6R binding in a Ca-dependent manner. When

hsIL-6R was administered in combination with 6RL#9-IgG1 or FH4-IgG1, the plasma hsIL-6R concentration on Day 1 could be reduced by 39 times or twice, respectively, in comparison to when hsIL-6R was administered in combination with H54/L28-IgG1. This demonstrates that calcium-dependent binding antibodies can accelerate the elimination of an antigen from the plasma.

[Example 6] Trials to improve the antigen elimination-accelerating effect of antibody with Ca-dependent antigen-binding (preparation of antibodies)

(6-1) Regarding the binding of IgG antibody to FcRn

IgG antibodies have longer plasma retention time as a result of FcRn binding. The binding between IgG and FcRn is observed only under an acidic condition (pH 6.0). By contrast, the binding is almost undetectable under a neutral condition (pH 7.4). An IgG antibody is taken up into cells in a nonspecific manner. The antibody returns to the cell surface by binding to endosomal FcRn under the endosomal acidic condition, and then dissociates from FcRn under the plasma neutral condition. When the FcRn binding under the acidic condition is lost by introducing mutations into the IgG Fc domain, the antibody retention time in plasma is markedly impaired because the antibody no longer recycles to the plasma from the endosome.

A reported method for improving the plasma retention of an IgG antibody is to enhance the FcRn binding under acidic conditions. Amino acid mutations are introduced into its Fc domain of an IgG antibody to improve its FcRn binding under acidic conditions. This increases the efficiency of recycling to the plasma from the endosome, resulting in improvement of the plasma retention. An important requirement in the amino acid substitution is not to augment the FcRn binding under neutral conditions. If an IgG antibody binds to FcRn under neutral conditions, the antibody does not dissociate from FcRn under the plasma neutral condition even if it returns to the cell surface by binding to FcRn under the endosomal acidic condition. In this case, the plasma retention is rather lost because the IgG antibody is not recycled to the plasma.

For example, as described in J Immunol. (2002) 169(9): 5171-80, an IgG1 antibody modified by introduction of amino acid substations so that the resulting antibody is capable of binding to mouse FcRn under a neutral condition (pH 7.4) was reported to exhibit very poor plasma retention when administered to mice. Furthermore, as described in J Immunol. (2009) 182(12): 7663-71; J Biol Chem. 2007 Jan. 19, 282(3): 1709-17; and J Immunol. 2002 Nov. 1, 169(9): 5171-80, an IgG1 antibody has been modified by introduction of amino acid substitutions so that the resulting antibody exhibits improved human FcRn binding under an acidic condition (pH 6.0) and at the same time becomes capable of binding to human FcRn under a neutral condition (pH 7.4). The resulting antibody was reported to show neither improvement nor alteration in plasma retention when administered to cynomolgus monkeys. Thus, the

antibody engineering technology for improving antibody functions has only focused on the improvement of antibody plasma retention by enhancing human FcRn binding under acidic conditions without enhancing it under a neutral condition (pH 7.4). To date, there is no report describing the advantage of improving human FcRn binding under a neutral condition (pH 7.4) by introducing amino acid substitutions into the Fc domain of an IgG antibody.

Antibodies that bind to antigens in a pH-dependent manner accelerate the elimination of soluble antigen. The antibodies produce the effect by repeatedly binding to soluble antigens multiple times. Thus, such antibodies are very useful. A method for augmenting FcRn binding under a neutral condition (pH 7.4) was tested to further enhance the antigen elimination-facilitating effect.

(6-2) Preparation of Ca-dependent human IL-6 receptor-binding antibodies having FcRn-binding activity under neutral conditions

Amino acid mutations to enhance FcRn binding under a neutral condition (pH 7.4) were introduced into FH4-IgG1 and 6RL#9-IgG1 which have a calcium-dependent antigen-binding ability, and H54/L28-IgG1 as a control which does not have the calcium-dependent antigen-binding ability. Amino acid mutations were introduced by a PCR method known to those skilled in the art. Specifically, FH4-N434W (heavy chain SEQ ID NO: 7; light chain SEQ ID NO: 8), 6RL#9-N434W (heavy chain SEQ ID NO: 9; light chain SEQ ID NO: 10), and H54/L28-N434W (heavy chain SEQ ID NO: 11; light chain SEQ ID NO: 12) were constructed by substituting Trp for Asn at position 434 in the EU numbering system in the heavy chain constant region of IgG1. The method for substituting an amino acid is as follows. Mutants were prepared using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) by the method described in the appended instruction manual. The resulting plasmid fragments were inserted into animal cell expression vectors to construct desired expression vectors. Antibody expression and purification, and determination of their concentrations were carried out by the methods described in Example 2.

[Example 7] Assessment of the elimination-accelerating effect of Ca-dependent binding antibodies using normal mice

(7-1) *In vivo* test using normal mice

Normal mice (C57BL/6J mouse; Charles River Japan) were administered with hsIL-6R (soluble human IL-6 receptor: prepared as described in REFERENCE EXAMPLE 1) alone or in combination with an anti-human IL-6 receptor antibody, and then assessed for the *in vivo* dynamics of hsIL-6R and the anti-human IL-6 receptor antibody. An hsIL-6R solution (5 μg/ml) or a mixed solutions of hsIL-6R and an anti-human IL-6 receptor antibody was administered at

10 mL/kg once into the caudal vein. The anti-human IL-6 receptor antibodies used were the above-described H54/L28-N434W, 6RL#9-N434W, and FH4-N434W.

The concentration of hsIL-6R was 5 μg/mL in all the mixed solutions. Meanwhile, the anti-human IL-6 receptor antibody concentration differs with each antibody. The concentrations of H54/L28-N434W, 6RL#9-N434W, and FH4-N434W were 0.042, 0.55, and 1 mg/ml, respectively. In this case, the anti-human IL-6 receptor antibody is present in excess over hsIL-6R in the mixed solutions, and therefore almost every hsIL-6R is assumed to be bound by the antibody. Blood was collected 15 minutes, 7 hours, 1 day, 2 days, 4 days, 7 days, 14 days, 21 days, and 28 days after administration. The collected blood was immediately centrifuged at 4°C and 12,000 rpm for 15 minutes to separate plasma. The separated plasma was stored in a freezer at -20°C or below before assay.

(7-2) ELISA measurement of the anti-human IL-6 receptor antibody concentration in plasma in normal mice

The anti-human IL-6 receptor antibody concentration in mouse plasma was measured by ELISA in the same manner as described in EXAMPLE 6. Time courses of the plasma concentrations of antibodies H54/L28-N434W, 6RL#9-N434W, and FH4-N434W in normal mice after intravenous administration determined by this method are shown in Fig. 12.

(7-3) Measurement of the plasma hsIL-6R concentration by electrochemiluminescence assay

The hsIL-6R concentration in mouse plasma was measured by the electrochemiluminescence method. hsIL-6R calibration curve samples adjusted to concentrations of 2,000, 1,000, 500, 250, 125, 62.5, and 31.25 pg/mL and mouse plasma assay samples diluted 50-fold or more were prepared. The samples were mixed with a solution of monoclonal anti-human IL-6R antibody (R&D) ruthenium-labeled with SULFO-TAG NHS Ester (Meso Scale Discovery) and biotinylated anti-human IL-6R antibody (R&D), and then allowed to react overnight at 4°C. The assay buffer used for the reaction contains 10 mM EDTA for the purpose of reducing the free Ca concentration in the samples so that almost every hsIL-6R dissociates from 6RL#9-N434W or FH4-N434W in the samples and exists in a free form. Then, the mixtures were aliquoted into the MA400 PR Streptavidin Plate (Meso Scale Discovery). After one hour of reaction at 25°C, the plate was washed. Immediately after Read Buffer T(x4) (Meso Scale Discovery) was aliquoted into the plate, measurement was carried out using the SECTOR PR 400 reader (Meso Scale Discovery). The hsIL-6R concentrations were calculated based on the response in the calibration curve using the analytical software, SOFTmax PRO (Molecular Devices). Time courses of the plasma hsIL-6R concentration in normal mice after intravenous administration determined by the above-described method are shown in Fig. 13.

The findings described above demonstrated that the FcRn binding at pH 7.4 was enhanced but, when hsIL-6R was simultaneously administered with a general antibody H54/L28-N434W, which does not exhibit Ca-dependent hsIL-6R binding, the elimination of hsIL-6R was considerably retarded as compared to when hsIL-6R was administered alone. Meanwhile, when hsIL-6R was simultaneously administered with 6RL#9-N434W or FH4-N434W which are antibodies that have enhanced FcRn binding at pH 7.4 and 100 times or higher hsIL-6R binding depending on Ca, the elimination of hsIL-6R was significantly accelerated as compared to when hsIL-6R was administered alone. When hsIL-6R was simultaneously administered with 6RL#9-N434W and FH4-N434W, the plasma hsIL-6R concentration on Day 1 could be reduced by 3 and 8 times, respectively, as compared to when hsIL-6R was administered alone. This demonstrates that the elimination of an antigen from the plasma can be further accelerated by enhancing the FcRn-binding ability of a calcium-dependent binding antibody at pH 7.4.

In comparison to a general antibody H54/L28-IgG1 which does not exhibit Ca-dependent hsIL-6R binding, antibody 6RL#9-IgG1 or FH4-IgG1 which has 100 times or higher Ca-dependent hsIL-6R binding were confirmed to have the effect to enhance the hsIL-6R elimination. Furthermore, in comparison to when hsIL-6R alone was administered, hsIL-6R and antibody 6RL#9-N434W or FH4-N434W which exhibits enhanced FcRn binding at pH 7.4 and has 100 times or higher hsIL-6R binding depending on Ca were confirmed to be able to accelerate hsIL-6R elimination. The data described above suggests that similar to an antibody that binds to an antigen in a pH-dependent manner, an antibody that binds to an antigen in a Ca-dependent manner dissociates from the antigen in the endosome, as illustrated in Fig. 1. As described in Example 1, there are limited types of epitopes targeted by antibodies with pH-dependent antigen binding (Fig. 3). However, by using antibodies with Ca-dependent antigen binding as revealed in the present invention, it is considered that one can expand the variety of epitopes to be targeted by antibodies capable of endosome-dependent antigen dissociation (Figs. 4 and 5).

[Example 8] Identification of calcium ion-binding site in antibody 6RL#9 by X-ray crystallography

(8-1) X-ray crystallography

As described in Example 4, the measurements of thermal denaturation temperature Tm suggested that antibody 6RL#9 binds to calcium ion. However, it was unpredictable which portion of antibody 6RL#9 binds to calcium ion. Then, by using the technique of X-ray crystallography, residues of antibody 6RL#9 that interact with calcium ion were identified.

(8-2) Expression and purification of antibody 6RL#9

Antibody 6RL#9 was expressed and purified for X-ray crystallography. Specifically, animal expression plasmids constructed to be capable of expressing the heavy chain (SEQ ID NO: 1) and light chain (SEQ ID NO: 2) of antibody 6RL#9 were introduced transiently into animal cells. The constructed plasmids were introduced by the lipofection method into cells of human fetal kidney cell-derived FreeStyle 293-F (Invitrogen) suspended in 800 ml of the FreeStyle 293 Expression Medium (Invitrogen) (final cell density: 1 x 10^6 cells/mL). The plasmid-introduced cells were cultured in a CO_2 incubator (37°C, 8% CO_2, 90 rpm) for five days. From the culture supernatant obtained as described above, antibodies were purified by a method known to those skilled in the art using the rProtein A Sepharose™ Fast Flow (Amersham Biosciences). Absorbance at 280 nm of purified antibody solutions was measured using a spectrophotometer. Antibody concentrations were calculated from the measured values using an extinction coefficient calculated by the PACE method (Protein Science (1995) 4, 2411-2423).

(8-3) Purification of antibody 6RL#9 Fab fragment

Antibody 6RL#9 was concentrated to 21 mg/ml using an ultrafilter with a molecular weight cutoff of 10,000 MWCO. A 5 mg/mL antibody sample (2.5 mL) was prepared by diluting the antibody solution using 4 mM L-cysteine/5 mM EDTA/20 mM sodium phosphate buffer (pH 6.5). 0.125 mg of papain (Roche Applied Science) was added to the sample. After stirring, the sample was incubated at 35°C for two hours. After incubation, a tablet of Protease Inhibitor Cocktail Mini, EDTA-free (Roche Applied Science) was dissolved in 10 ml of 25 mM MES buffer (pH 6) and added to the sample. The sample was incubated on ice to stop the papain proteolytic reaction. Then, the sample was loaded onto a 1-ml cation-exchange column HiTrap SP HP (GE Healthcare) equilibrated with 25 mM MES buffer (pH 6), downstream of which a 1-ml HiTrap MabSelect Sure Protein A column (GE Healthcare) was connected in tandem. A purified fraction of the Fab fragment of antibody 6RL#9 was obtained by performing elution with a linear NaCl concentration gradient up to 300 mM in the above-described buffer. Then, the resulting purified fraction was concentrated to about 0.8 ml using a 5000 MWCO ultrafilter. The concentrate was loaded onto a gel filtration column Superdex 200 10/300 GL (GE Healthcare) equilibrated with 100 mM HEPES buffer (pH 8) containing 50 mM NaCl. The purified Fab fragment of antibody 6RL#9 for crystallization was eluted from the column using the same buffer. All the column treatments described above were carried out at a low temperature of 6 to 7.5°C.

(8-4) Crystallization of the antibody 6RL#9 Fab fragment in the presence of Ca

Seed crystals of the 6RL#9 Fab fragment were prepared in advance under general conditions. Then, the purified Fab fragment of antibody 6RL#9 in 5 mM $CaCl_2$ was

concentrated to 12 mg/ml with a 5000 MWCO ultrafilter. Next, the sample concentrated as described above was crystallized by the hanging drop vapor diffusion method using 100 mM HEPES buffer (pH 7.5) containing 20% to 29% PEG4000 as a reservoir solution. The above-described seed crystals were crushed in 100 mM HEPES buffer (pH 7.5) containing 29% PEG4000 and 5 mM $CaCl_2$, and serially diluted to 100 to 10,000 folds. Then, 0.2 μL of diluted solutions were combined with a mixture of 0.8 μl of the reservoir solution and 0.8 μl of the concentrated sample to prepare crystallization drops on a glass cover slide. The crystal drops were allowed to stand at 20°C for two to three days to prepare thin plate-like crystals. X-ray diffraction data were collected using the crystals.

(8-5) Crystallization of the antibody 6RL#9 Fab fragment in the absence of Ca

The purified Fab fragment of antibody 6RL#9 was concentrated to 15 mg/ml using a 5000 MWCO ultrafilter. Then, the sample concentrated as described above was crystallized by the hanging drop vapor diffusion method using 100 mM HEPES buffer (pH 7.5) containing 18% to 25% PEG4000 as a reservoir solution. Crystals of the antibody 6RL#9 Fab fragment obtained in the presence of Ca were crushed in 100 mM HEPES buffer (pH 7.5) containing 25% PEG4000, and serially diluted to 100 to 10,000 folds. Then, 0.2 μL of diluted solutions were combined with a mixture of 0.8 μl of the reservoir solution and 0.8 μl of the concentrated sample to prepare crystallization drops on a glass cover slide. The crystal drops were allowed to stand at 20°C for two to three days to prepare thin plate-like crystals. X-ray diffraction data were collected using the crystals.

(8-6) X-ray crystallographic measurement of Fab fragment crystal from antibody 6RL#9 in the presence of Ca

Crystals of the Fab fragment of antibody 6RL#9 prepared in the presence of Ca were soaked in 100 mM HEPES buffer (pH 7.5) solution containing 35% PEG4000 and 5 mM $CaCl_2$. By removing the exterior solution from the surface of a single crystal with a micro-nylon-loop pin, the single crystal was frozen in liquid nitrogen. X-ray diffraction data of the frozen crystal was collected from beam line BL-17A of the Photon Factory in the High Energy Accelerator Research Organization. The frozen crystal was maintained in the frozen state during the measurement by constantly placing it in a stream of nitrogen gas at -178°C. A total of 180 diffraction images were collected using the CCD detector Quantum315r (ADSC) attached to the beam line while rotating the crystal in 1° intervals. Lattice constant determination, diffraction spot indexing, and diffraction data analysis were performed using programs Xia2 (CCP4 Software Suite), XDS Package (Walfgang Kabsch), and Scala (CCP4 Software Suite). Finally, diffraction intensity data up to 2.2 angstrom resolution was obtained. The crystal belongs to

space group P212121 with lattice constant a = 45.47 angstrom, b = 79.86 angstrom, c = 116.25 angstrom, $\alpha = 90°$, $\beta = 90°$, and $\gamma = 90°$.

(8-7) X-ray crystallographic measurement of the Fab fragment crystal from antibody 6RL#9 in the absence of Ca

Crystals of the Fab fragment of antibody 6RL#9 prepared in the absence of Ca were soaked in 100 mM HEPES buffer (pH 7.5) solution containing 35% PEG4000. By removing the exterior solution from the surface of a single crystal with a micro-nylon-loop pin, the single crystal was frozen in liquid nitrogen. X-ray diffraction data of the frozen crystal was collected from beam line BL-5A of the Photon Factory in the High Energy Accelerator Research Organization. The frozen crystal was maintained in the frozen state during the measurement by constantly placing it in a stream of nitrogen gas at -178°C. A total of 180 diffraction images were collected using the CCD detector Quantum210r (ADSC) attached to the beam line while rotating the crystal in 1° intervals. Lattice constant determination, diffraction spot indexing, and diffraction data analysis were performed using programs Xia2 (CCP4 Software Suite), XDS Package (Walfgang Kabsch), and Scala (CCP4 Software Suite). Finally, diffraction intensity data up to 2.3 angstrom resolution was obtained. The crystal belongs to space group P212121 with lattice constant a = 45.40 angstrom, b = 79.63 angstrom, c = 116.07 angstrom, $\alpha = 90°$, $\beta = 90°$, $\gamma = 90°$, and thus is structurally identical to the crystal prepared in the presence of Ca.

(8-8) X-ray crystallographic measurement of the Fab fragment crystal from antibody 6RL#9 in the presence of Ca

The crystal structure of the antibody 6RL#9 Fab fragment in the presence of Ca was determined by a molecular replacement method using the Phaser program (CCP4 Software Suite). The number of molecules in an asymmetrical unit was estimated to be one from the size of crystal lattice and molecular weight of the antibody 6RL#9 Fab fragment. Based on the primary sequence homology, a portion of amino acid positions 112 to 220 from A chain and a portion of amino acid positions 116 to 218 from B chain in the conformational coordinate of PDB code 1ZA6 were used as model molecules for analyzing the CL and CH1 regions. Then, a portion of amino acid positions 1 to 115 from B chain in the conformational coordinate of PDB code 1ZA6 was used as a model molecule for analyzing the VH region. Finally, a portion of amino acid positions 3 to 147 of the light chain in the conformational coordinate of PDB code 2A9M was used as a model molecule for analyzing the VL region. Based on this order, an initial structure model for the antibody 6RL#9 Fab fragment was obtained by determining from translation and rotation functions the positions and orientations of the model molecules for analysis in the crystal lattice. The crystallographic reliability factor R for the reflection data at

25 to 3.0 angstrom resolution was 46.9% and Free R was 48.6% after rigid body refinement where the VH, VL, CH1, and CL domains were each allowed to deviate from the initial structure model. Then, model refinement was achieved by repeating structural refinement using program Refmac5 (CCP4 Software Suite) followed by model revision performed using program Coot (Paul Emsley) with reference to the Fo-Fc and 2Fo-F electron density maps where the coefficients Fo-Fc and 2Fo-Fc were calculated using experimentally determined structural factor Fo, structural factor Fc calculated based on the model, and the phases. The final refinement was carried out using program Refmac5 (CCP4 Software Suite) based on the Fo-Fc and 2Fo-F electron density maps by adding water molecule and Ca ion into the model. With 21,020 reflection data at 25 to 2.2 angstrom resolution, eventually the crystallographic reliability factor R became 20.0% and free R became 27.9% for the model consisting of 3440 atoms.

(8-9) Measurement of X-ray diffraction data of the Fab fragment crystal from antibody 6RL#9 in the absence of Ca

The crystal structure of the antibody 6RL#9 Fab fragment in the absence of Ca was determined based on the structure of the crystal prepared in the presence of Ca. Water and Ca ion molecules were omitted from the conformational coordinate of the crystal of the antibody 6RL#9 Fab fragment prepared in the presence of Ca. The crystallographic reliability factor R for the data of reflection at 25 to 3.0 angstrom resolution was 30.3% and Free R was 31.7% after the rigid body refinement where the VH, VL, CH1, and CL domains were each allowed to deviate. Then, model refinement was achieved by repeating structural refinement using program Refmac5 (CCP4 Software Suite) followed by model revision performed using program Coot (Paul Emsley) with reference to the Fo-Fc and 2Fo-Fc electron density maps where the coefficients Fo-Fc and 2Fo-Fc were calculated using experimentally determined structural factor Fo, structural factor Fc calculated based on the model, and the phases. The final refinement was carried out using program Refmac5 (CCP4 Software Suite) based on the Fo-Fc and 2Fo-F electron density maps by adding water molecule and Ca ion into the model. With 18,357 reflection data at 25 to 2.3 angstrom resolution, eventually the crystallographic reliability factor R became 20.9% and free R became 27.7% for the model consisting of 3351 atoms.

(8-10) Comparison of X-ray crystallographic diffraction data of the Fab fragments of antibody 6RL#9 between in the presence and absence of Ca

When the crystallographic structures of the Fab fragments of antibody 6RL#9 are compared between in the presence and absence of Ca, significant changes are seen in the heavy chain CDR3. The structure of the heavy chain CDR3 of the antibody 6RL#9 Fab fragment determined by X-ray crystallography is shown in Fig. 14. Specifically, a calcium ion resided at

the center of the heavy chain CDR3 loop region of the antibody 6RL#9 Fab fragment prepared in the presence of Ca. The calcium ion was assumed to interact with positions 95, 96, and 100a (Kabat's numbering) of the heavy chain CDR3. It was believed that the heavy chain CDR3 loop which is important for the antigen binding was stabilized by calcium binding in the presence of Ca, and became an optimum structure for antigen binding. There is no report demonstrating that calcium binds to the antibody heavy chain CDR3. Thus, the calcium-bound structure of the antibody heavy chain CDR3 is a novel structure. The heavy chain CDR3 is known to be most important for antigen binding. The motif for which calcium ion is required for maintaining the structure of the heavy chain CDR3, revealed as described in the present Example, implies that calcium ion plays an important role in antigen binding. Specifically, it is highly plausible that antibodies with this motif bind to an antigen in a calcium ion-dependent manner. For example, when a synthetic library having this motif is prepared, one can efficiently isolate calcium-dependent binding antibodies from the library.

[Example 9] Preparation of antibodies that bind to IL-6 in a Ca-dependent manner from a human antibody library using phage display techniques

(9-1) Construction of a phage display library of naïve human antibodies

A human antibody phage display library containing multiple phages that display various human antibody Fab domain sequences was constructed by a method known to those skilled in the art using, as a template, polyA RNA prepared from human PBMC, commercially available human polyA RNA, and such.

(9-2) Preparation of antibody fragments that bind to the antigen in a Ca-dependent manner from library by bead panning

Primary selection from the constructed phage display library of naïve human antibodies was carried out by enriching antibody fragments that have antigen (IL-6)-binding activity. The antigen used was biotin-labeled IL-6.

Phages were produced from *E. coli* carrying the constructed phagemid for phage display. To precipitate the phages produced by *E. coli*, 2.5 M NaCl/10% PEG was added to the *E. coli* culture medium. The phage fraction was diluted with TBS to prepare a phage library solution. Then, BSA and $CaCl_2$ were added the phage library solution at final concentrations of 4% and 1.2 mM calcium ion concentration, respectively. The panning method used was a conventional panning method using antigen-immobilized magnetic beads (J. Immunol. Methods. (2008) 332(1-2): 2-9; J. Immunol. Methods. (2001) 247(1-2): 191-203; Biotechnol. Prog. (2002) 18(2): 212-20; Mol. Cell Proteomics (2003) 2(2): 61-9). The magnetic beads used were NeutrAvidin-coated beads (Sera-Mag SpeedBeads NeutrAvidin-coated) and Streptavidin-coated beads

(Dynabeads M-280 Streptavidin).

Specifically, 250 pmol of the biotin-labeled antigen was added to the prepared phage library solution. Thus, the solution was contacted with the antigen at room temperature for 60 minutes. Magnetic beads blocked with BSA were added, and the antigen-phage complex was allowed to bind to the magnetic beads at room temperature for 15 minutes. The beads were washed three times with 1.2 mM $CaCl_2$/TBST (TBST containing 1.2 mM $CaCl_2$), and then twice with 1 ml of 1.2 mM $CaCl_2$/TBS (TBS containing 1.2 mM $CaCl_2$). Thereafter, 0.5 ml of 1 mg/ml trypsin was added to the beads. After 15 minutes of dispersion at room temperature, the beads were immediately separated using a magnetic stand to collect a phage suspension. The prepared phage suspension was added to 10 ml of *E. coli* of stain TG1 at the logarithmic growth phase (OD600 = 0.4 to 0.5). The *E. coli* was incubated with gentle stirring at 37°C for one hour to infect the phages. The infected *E. coli* was seeded in a plate (225 mm x 225 mm). Then, phages were collected from the culture medium of the seeded *E. coli* to prepare a phage library solution.

In the second round and subsequent panning, phages were enriched using the Ca-dependent binding activity as an indicator. Specifically, 40 pmol of the biotin-labeled antigen was added to the prepared phage library solution. Thus, the phage library was contacted with the antigen at room temperature for 60 minutes. Magnetic beads blocked with BSA were added, and the antigen-phage complex was allowed to bind to the magnetic beads at room temperature for 15 minutes. The beads were washed with 1 ml of 1.2 mM $CaCl_2$/TBST and 1.2 mM $CaCl_2$/TBS. Next, 0.1 ml of 2 mM EDTA/TBS was added to the beads. After dispersion at room temperature, the beads were immediately separated using a magnetic stand to collect a phage suspension. The pIII protein (helper phage-derived protein pIII) was cleaved from phages that did not display Fab by adding 5 µl of 100 mg/ml trypsin to the collected phage suspension to eliminate the ability of phages displaying no Fab to infect *E. coli*. Phages collected from the trypsinized liquid phage stock was added to 10 ml of *E. coli* cells of the TG1 strain at the logarithmic growth phase (OD600 = 0.4 to 0.7). The *E. coli* was incubated while gently stirring at 37°C for one hour to infect phage. The infected *E. coli* was seeded in a plate (225 mm x 225 mm). Then, phages were collected from the culture medium of the seeded *E. coli* to prepare a liquid stock of phage library. Panning was performed three times using the Ca-dependent binding activity as an indicator.

(9-3) Assessment by phage ELISA

Culture supernatants containing phages were collected from single colonies of *E. coli* obtained by the method described above according to a conventional method (Methods Mol. Biol. (2002) 178, 133-145). BSA and $CaCl_2$ were added at final concentrations of 4% and 1.2 mM calcium ion concentration, respectively, to the phage-containing culture supernatants. The

supernatants were subjected to ELISA by the following procedure. A StreptaWell 96-well microtiter plate (Roche) was coated overnight with 100 µl of PBS containing the biotin-labeled antigen. The antigen was removed by washing each well of the plate with PBST. Then, the wells were blocked with 250 µl of 4% BSA-TBS for one hour or more. After removal of 4% BSA-TBS, the prepared culture supernatants were added to the each well. The plate was incubated at 37°C for one hour so that the antibody-displaying phages were allowed to bind to the antigen on each well. After each well was washed with 1.2 mM $CaCl_2$/TBST, 1.2 mM $CaCl_2$/TBS or 1 mM EDTA/TBS was added. The plate was left for incubation at 37°C for 30 minutes. After washing with 1.2 mM $CaCl_2$/TBST, an HRP-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) diluted with TBS containing BSA and calcium ion at final concentrations of 4% and 1.2 mM calcium ion concentration was added to each well, and the plate was incubated for one hour. After washing with 1.2 mM $CaCl_2$/TBST, the TMB single solution (ZYMED) was added to each well. The chromogenic reaction in the solution of each well was stopped by adding sulfuric acid. Then, the developed color was assessed by measuring absorbance at 450 nm.

From the 96 clones isolated, antibodies 6KC4-1#85, 6LC4-1#15, and 6LC4-2#16 having Ca-dependent IL-6-binding activity were obtained by phage ELISA. Using antibody fragments that were predicted to have a Ca-dependent antigen-binding activity based on the result of the phage ELISA described above as a template, genes were amplified with specific primers and their sequences were analyzed. The heavy-chain and light-chain variable region sequences of antibody 6KC4-1#85 are shown in SEQ ID NOs: 25 and 26, respectively. The polynucleotide encoding the heavy-chain variable region of antibody 6KC4-1#85 (SEQ ID NO: 25) was linked to a polynucleotide encoding an IgG1-derived sequence (SEQ ID NO: 65) by PCR method. The resulting DNA fragment was inserted into an animal cell expression vector to construct an expression vector for the heavy chain of SEQ ID NO: 27. A polynucleotide encoding the light-chain variable region of antibody 6KC4-1#85 (SEQ ID NO: 26) was linked to a polynucleotide encoding the constant region of the natural Kappa chain (SEQ ID NO: 28) by PCR. A DNA fragment encoding the linked sequence shown in SEQ ID NO: 29 was inserted into an animal cell expression vector. Using the same method, antibody 6LC4-1#15 (heavy chain SEQ ID NO: 68; light chain SEQ ID NO: 69) and antibody 6LC4-2#16 (heavy chain SEQ ID NO: 70; light chain SEQ ID NO: 71) were also inserted into cell expression vectors. Sequences of the constructed variants were confirmed by a method known to those skilled in the art.

(9-4) Expression and purification of antibodies

Clones that were predicted to have a Ca-dependent antigen-binding activity based on the

result of phage ELISA were inserted into animal cell expression plasmids. Antibody expression was carried out by the following method. Cells of human fetal kidney cell-derived FreeStyle 293-F (Invitrogen) were suspended in the FreeStyle 293 Expression Medium (Invitrogen), and plated at a cell density of 1.33 x 10^6 cells/ml (3 ml) into each well of a 6-well plate. The prepared plasmids were introduced into cells by a lipofection method. The cells were cultured for four days in a CO_2 incubator (37°C, 8% CO_2, 90 rpm). From the culture supernatants, antibodies were purified using the rProtein A Sepharose™ Fast Flow (Amersham Biosciences) by a method known to those skilled in the art. Absorbance at 280 nm of the purified antibody solutions was measured using a spectrophotometer. Antibody concentrations were calculated from the determined values using an extinction coefficient calculated by the PACE method (Protein Science (1995) 4: 2411-2423).

(9-5) Binding assay of calcium-dependent anti-IL6 antibodies

Using Biacore T100 (GE Healthcare), the prepared antibodies were assessed for their binding activity (dissociation constant K_D (M)) to human interleukin 6 (hIL6) at pH 7.4. The measurement was carried out using as a running buffer 0.05% Tween20, 10 mmol/l ACES, 150 mmol/l NaCl (pH 7.4) containing 3 µM or 1.2 mM $CaCl_2$.

After an adequate amount of recombinant Protein A/G (Thermo Scientific) was immobilized onto Sensor chip CM5 (GE Healthcare) by an amino coupling method, antibodies were allowed to bind thereto. An appropriate concentration of hIL6 (human interleukin 6; Kamakura Techno-Science, Inc.) was injected as an analyte to interact with antibodies on the sensor chip. Then, the sensor chip was regenerated by injecting 10 mmol/l glycine-HCl (pH 1.5). The measurement was carried out at 37°C. The sensorgram resulting from the measurement is shown in Fig. 15. The result demonstrates that antibodies 6LC4-1#15-IgG1, 6LC4-2#16-IgG1, and 6KC4-1#85-IgG1 had weaker hIL6 binding under the condition of 3 µM Ca^{2+} concentration than at 1.2 mM. The finding described above suggests that this method is applicable to other antigens since the property of calcium-dependent antigen binding was proven for IL-6 as well as for IL-6R demonstrated in Example 3.

[Example 10] Assessment of antibody 6KC4-1#85 for calcium ion binding

(10-1) Assessment of antibody 6KC4-1#85 for calcium ion binding

Calcium-dependent antigen-binding antibody 6KC4-1#85 which was isolated from a human antibody library was assessed for its calcium binding. Whether the measured Tm value varies depending on the ionized calcium concentration condition was assessed by the method described in Example 4.

Tm values for the Fab domain of antibody 6KC4-1#85 are shown in Table 11. As

shown in Table 11, the Tm value of the 6KC4-1#85 antibody Fab domain varied depending on the calcium ion concentration. This demonstrates that antibody 6KC4-1#85 binds to calcium.

[Table 11]

ANTIBODY	CALCIUM ION CONCENTRATION		Δ Tm (°C)
	3 μ M	2 mM	2 mM−3 μ M
6KC4−1#85	71.49	75.39	3.9

(10-2) Identification of calcium ion-binding site in antibody 6KC4-1#85

As demonstrated in (10-1) of Example 10, antibody 6KC4-1#85 binds to calcium ion. However, 6KC4-1#85m does not have a calcium-binding motif such as the hVk5-2 sequence described below. Thus, to identify residues responsible for the calcium ion binding of antibody 6KC4-1#85, altered heavy chains (6_H1-11 (SEQ ID NO: 30), 6_H1-12 (SEQ ID NO: 31), 6_H1-13 (SEQ ID NO: 32), 6_H1-14 (SEQ ID NO: 33), 6_H1-15 (SEQ ID NO: 34)) and altered light chains (6_L1-5 (SEQ ID NO: 35) and 6_L1-6 (SEQ ID NO: 36)) were constructed by substituting an Asp (D) residue in the CDR of antibody 6KC4-1#85 with an Ala (A) residue which does not participate in the binding or chelation of calcium ion. By the method described in Example 2, altered antibodies were purified from the culture supernatants of animal cells introduced with expression vectors carrying the altered antibody genes. The purified altered antibodies were assessed for their calcium binding by the method described in Example 4. The measurement result is shown in Table 12.

[Table 12]

HEAVY CHAIN	LIGHT CHAIN	ALTERED RESIDUE	CALCIUM ION CONCENTRATION		ΔTm (℃)
			3 μM	2 mM	2 mM-3 μM
6KC4-1#85	6KC4-1#85	WILD-TYPE	71.49	75.39	3.9
6H1-11	6KC4-1#85	H CHAIN POSITION 61 (Kabat NUMBERING)	71.73	75.56	3.83
6H1-12	6KC4-1#85	H CHAIN POSITION 95 (Kabat NUMBERING)	72.9	73.43	0.53
6H1-13	6KC4-1#85	H CHAIN POSITION 100a (Kabat NUMBERING)	70.94	76.25	5.31
6H1-14	6KC4-1#85	H CHAIN POSITION 100g (Kabat NUMBERING)	73.95	75.14	1.19
6H1-15	6KC4-1#85	H CHAIN POSITION 101 (Kabat NUMBERING)	65.37	66.25	0.87
6KC4-1#85	6L1-5	L CHAIN POSITION 50 (Kabat NUMBERING)	71.92	76.08	4.16
6KC4-1#85	6L1-6	L CHAIN POSITION 92 (Kabat NUMBERING)	72.13	78.74	6.61

As shown in Table 12, substitution of an Ala residue for the residue at position 95 or 101 (Kabat's numbering) in the heavy chain CDR3 of antibody 6KC4-1#85 resulted in loss of the calcium-binding activity of antibody 6KC4-1#85. This suggests that these residues are responsible for calcium binding. It was demonstrated that the calcium-binding motif around the base of the loop of the heavy chain CDR3 in antibody 6KC4-1#85, which was identified based on the calcium-binding activity of antibodies altered from antibody 6KC4-1#85, could also be used as a calcium-binding motif in the antigen-binding domain of an antigen-binding molecule of the present invention. Like the motif revealed as described in Example 8, this calcium-binding motif is located in the heavy chain CDR3. Thus, likewise, for example, when a synthetic library having this motif is constructed, calcium-dependent binding antibodies can be efficiently isolated from the library.

[Example 11] Search for human germline sequences that bind to calcium ion

(11-1) Isolation of human germline sequences

Calcium ion-binding antibodies containing human germline sequences have not been reported. Thus, the germline sequences of antibodies having human germline sequences were cloned using as a template cDNA prepared from Human Fetal Spleen Poly RNA (Clontech) to assess whether antibodies having human germline sequences bind to calcium ion. Cloned DNA fragments were inserted into animal cell expression vectors. The nucleotide sequences of the constructed expression vectors were determined by a method known to those skilled in the art. The SEQ IDs are shown in Table 13. By PCR, polynucleotides encoding SEQ ID NO: 37 (Vk1), SEQ ID NO: 38 (Vk2), SEQ ID NO: 39 (Vk3), SEQ ID NO: 40 (Vk4), and SEQ ID NO: 41 (Vk5) were linked to a polynucleotide encoding the natural Kappa chain constant region (SEQ ID NO: 28). The linked DNA fragments were inserted into animal cell expression vectors. Furthermore, polynucleotides encoding SEQ ID NO: 42 (Vk1), SEQ ID NO: 43 (Vk2), SEQ ID NO: 44 (Vk3), SEQ ID NO: 45 (Vk4), and SEQ ID NO: 46 (Vk5) were linked by PCR to a polynucleotide encoding a polypeptide (SEQ ID NO: 65) having a deletion of two amino acids at the C terminus of IgG1. The resulting DNA fragments were inserted into animal cell expression vectors. The sequences of the constructed variants were confirmed by a method known to those skilled in the art.

[Table 13]

LIGHT CHAIN GERMLINE SEQUENCE	HEAVY CHAIN VARIABLE REGION SEQ ID NO	LIGHT CHAIN VARIABLE REGION SEQ ID NO
Vk1	42	37
Vk2	43	38
Vk3	44	39
Vk4	45	40
Vk5	46	41

(11-2) Expression and purification of antibodies

The constructed animal cell expression vectors inserted with the DNA fragments having the five types of human germ-line sequences were introduced into animal cells. Antibody expression was carried out by the following method. Cells of human fetal kidney cell-derived FreeStyle 293-F (Invitrogen) were suspended in the FreeStyle 293 Expression Medium (Invitrogen), and plated at a cell density of 1.33×10^6 cells/ml (3 ml) into each well of a 6-well plate. The prepared plasmids were introduced into cells by a lipofection method. The cells were cultured for four days in a CO_2 incubator (37°C, 8% CO_2, 90 rpm). From the culture

supernatants prepared as described above, antibodies were purified using the rProtein A Sepharose™ Fast Flow (Amersham Biosciences) by a method known to those skilled in the art. Absorbance at 280 nm of the purified antibody solutions was measured using a spectrophotometer. Antibody concentrations were calculated from the determined values using an extinction coefficient calculated by the PACE method (Protein Science (1995) 4: 2411-2423).

(11-3) Assessment of antibodies having human germ-line sequences for their calcium ion-binding activity

The purified antibodies were assessed for their calcium ion-binding activity. The purified antibodies were dialyzed (EasySEP, TOMY) against a solution containing 20 mM Tris-HCl, 150 mM NaCl, and 2 mM $CaCl_2$ (pH 7.4), or 20 mM Tris-HCl, 150 mM NaCl, and 3 μM $CaCl_2$ (pH 7.4). The antibody solutions as a test substance were adjusted to 0.1 mg/ml using the same solution used for dialysis, and DSC measurement was carried out at a rate of temperature increase of 240°C/hr from 20 to 115°C. Based on the obtained DSC denaturation curves, the midpoint temperature of thermal denaturation (Tm value) was calculated for the Fab domain of each antibody. The Tm values are shown in Table 14.

[Table 14]

LIGHT CHAIN GERMLINE SEQUENCE	CALCIUM ION CONCENTRATION		ΔTm (℃)
	3 μ M	2 mM	2 mM-3 μ M
Vk1	80.32	80.78	0.46
Vk2	80.67	80.61	-0.06
Vk3	81.64	81.36	-0.28
Vk4	70.74	70.74	0
Vk5	71.52	74.17	2.65

The result showed that the Tm values of the Fab domains of antibodies having the hVk1, hVk2, hVk3, or hVk4 sequence did not vary depending on the calcium ion concentration in the Fab domain-containing solutions. Meanwhile, the Tm value for the antibody Fab domain having the hVk5 sequence varied depending on the calcium ion concentration in the Fab domain-containing solution. This demonstrates that the hVk5 sequence binds to calcium ion.

[Example 12] Assessment of the human Vk5 (hVk5) sequence

(12-1) hVk5 sequence

The only hVk5 sequence registered in Kabat's database is hVk5-2 sequence. Hereinafter, hVk5 and hVk5-2 are used synonymously.

(12-2) Construction, expression, and purification of a non-glycosylated form of the hVk5-2 sequence

The hVk5-2 sequence has a sequence for N glycosylation at position 20 amino acid (Kabat's numbering). Sugar chains attached to proteins exhibit heterogeneity. Thus, it is desirable to lose the glycosylation from the viewpoint of substance homogeneity. In this context, variant hVk5-2_L65 (SEQ ID NO: 47) in which the Asn (N) residue at position 20 (Kabat's numbering) is substituted with Thr (T) was constructed. Amino acid substitution was carried out by a method known to those skilled in the art using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). A DNA encoding the variant hVk5-2_L65 was inserted into an animal expression vector. The animal expression vector inserted with the constructed DNA encoding variant hVk5-2_L65, in combination with an animal expression vector having an insert to express CIM_H (SEQ ID NO: 48) as a heavy chain, was introduced into animal cells by the method described in Example 2. The antibody comprising hVk5-2_L65 and CIM_H, which was expressed in animal cells introduced with the vectors, was purified by the method described in Example 2.

(12-3) Assessment of the antibody having the non-glycosylated hVk5-2 sequence for physical properties

The isolated antibody having the modified sequence hVk5-2_L65 was analyzed by ion-exchange chromatography to test whether it is less heterogeneous than the antibody having the original sequence hVk5-2 before modification. The procedure of ion-exchange chromatography is shown in Table 15. The analysis result showed that hVk5-2_L65 modified at the glycosylation site was less heterogeneous than the original sequence hVk5-2, as shown in Fig. 16.

[Table 15]

	CONDITION
COLUMN	TOSOH TSKgel DEAE-NPR
MOBILE PHASE	A; 10 mM Tris-HCl, 3 μM $CaCl_2$(pH8.0)
	B; 10 mM Tris-HCl, 500 mM NaCl, 3 μM $CaCl_2$ (pH8.0)
GRADIENT SCHEDULE	%B = 0 - (5min) - 0 - 2%/1min
COLUMN TEMPERATURE	40℃
DETECTION	280 nm
INJECTION VOLUME	100 μL (5 μg)

Next, whether the less-heterogeneous hVk5-2_L65 sequence-comprising antibody binds to calcium ion was assessed by the method described in Example 4. The result showed that the Tm value for the Fab domain of the antibody having hVk5-2_L65 with altered glycosylation site also varied depending on the calcium ion concentration in the antibody solutions, as shown in Table 16. Specifically, it was demonstrated that the Fab domain of the antibody having hVk5-2_L65 with altered glycosylation site binds to calcium ion.

[Table 16]

LIGHT CHAIN	GLYCOSYLATED SEQUENCE	CALCIUM ION CONCENTRATION		ΔTm(℃)
		3 μM	2 mM	2 mM-3 μM
hVk5-2	YES	71.52	74.17	2.65
hVk5-2_L65	NO	71.51	73.66	2.15

[Example 13] Assessment of the calcium ion-binding activity of antibody molecules having CDR sequence of the hVk5-2 sequence

(13-1) Construction, expression, and purification of modified antibodies having a CDR sequence from the hVk5-2 sequence

The hVk5-2_L65 sequence is a sequence with altered amino acids at a glycosylation site in the framework of human Vk5-2 sequence. As described in Example 12, it was demonstrated that calcium ion bound even after alteration of the glycosylation site. Meanwhile, from the viewpoint of immunogenicity, it is generally desirable that the framework sequence is a germline sequence. Thus, the present inventors assessed whether an antibody framework sequence could be substituted with the framework sequence of a non-glycosylated germline sequence while maintaining the calcium ion-binding activity of the antibody.

Polynucleotides encoding chemically synthesized sequences which comprise an altered framework sequence of the hVk5-2 sequence, hVk1, hVk2, hVk3, or hVk4 (CaVk1 (SEQ ID NO: 49), CaVk2 (SEQ ID NO: 50), CaVk3 (SEQ ID NO: 51), or CaVk4 (SEQ ID NO: 52), respectively) were linked by PCR to a polynucleotide encoding the constant region (SEQ ID NO: 28) of the natural Kappa chain. The linked DNA fragments were inserted into animal cell expression vectors. Sequences of the constructed variants were confirmed by a method known to those skilled in the art. Each plasmid constructed as described above was introduced into animal cells in combination with a plasmid inserted with a polynucleotide encoding CIM_H (SEQ ID NO: 48) by the method described in Example 2. The expressed antibody molecules of interest were purified from culture media of the animal cells introduced with the plasmids.

(13-2) Assessment of altered antibodies having the CDR sequence of the hVk5-2 sequence for their calcium ion-binding activity

Whether calcium ion binds to altered antibodies having the CDR sequence of the hVk5-2 sequence and the framework sequences of germline sequences other than hVk5-2 (hVk1, hVk2, hVk3, and hVk4) was assessed by the method described in Example 4. The assessment result is shown in Table 17. The Tm value of the Fab domain of each altered antibody was revealed to vary depending on the calcium ion concentration in the antibody solutions. This demonstrates that antibodies having a framework sequence other than the framework sequences of the hVk5-2 sequence also bind to calcium ion. Specifically, it was demonstrated that the motif in the CDR sequence of the hVk5-2 sequence is responsible for the calcium ion binding while the framework can be any framework sequence.

[Table 17]

GERMLINE (LIGHT CHAIN FRAMEWORK SEQUENCE)	CALCIUM ION CONCENTRATION		ΔTm (℃)
	3 μM	2 mM	2 mM-3 μM
hVk1	77.51	79.79	2.28
hVk2	78.46	80.37	1.91
hVk3	77.27	79.54	2.27
hVk4	80.35	81.38	1.03
hVk5-2	71.52	74.17	2.65

The thermal denaturation temperature (Tm value), as an indicator of thermal stability, of the Fab domain of each antibody altered to have the CDR sequence of the hVk5-2 sequence and the framework sequence of a germ-line sequence other than the hVk5-2 sequence (hVk1, hVk2, hVk3, or hVk4) was demonstrated to be greater than that of the Fab domain of the original antibody having the hVk5-2 sequence. This result shows that antibodies having the CDR sequence of the hVk5-2 sequence and the framework sequence of hVk1, hVk2, hVk3, or hVk4 not only have calcium ion-binding activity but also are excellent molecules from the viewpoint of thermal stability.

[Example 14] Identification of the calcium ion-binding site in human germline hVk5-2 sequence

(14-1) Design of mutation site in the CDR sequence of the hVk5-2 sequence

As described in Example 13, antibodies having the light chain resulting from introduction of the CDR domain of the hVk5-2 sequence into the framework sequence of a different germline sequence were also demonstrated to bind to calcium ion. This result suggests that in hVk5-2 a calcium ion-binding site is localized within its CDR. Amino acids that bind to calcium ion, i.e., chelate calcium ion, include negatively charged amino acids and amino acids that can be a hydrogen bond acceptor. Thus, it was tested whether antibodies having a mutant hVk5-2 sequence with a substitution of an Ala (A) residue for an Asp (D) or Glu (E) residue in the CDR sequence of the hVk5-2 sequence bind to calcium ion.

(14-2) Construction of variant hVk5-2 sequences with Ala substitution, and expression and purification of antibodies

Antibody molecules were prepared to comprise a light chain with substitution of an Ala residue for Asp and/or Glu residue in the CDR sequence of the hVk5-2 sequence. As described in Example 12, non-glycosylated variant hVk5-2_L65 exhibited calcium ion binding and was assumed to be equivalent to the hVk5-2 sequence in terms of calcium ion binding. In this Example, amino acid substitutions were introduced into hVk5-2_L65 as a template sequence. Constructed variants are shown in Table 18. Amino acid substitutions were carried out by methods known to those skilled in the art such as using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), PCR, or the In fusion Advantage PCR Cloning Kit (TAKARA) to construct expression vectors for altered light chains having an amino acid substitution.

[Table 18]

LIGHT CHAIN VARIANT NAME	ALTERED POSITION (Kabat's NUMBERING)	SEQ ID NO
hVk5-2_L65	WILDTYPE	47
hVk5-2_L66	30	53
hVk5-2_L67	31	54
hVk5-2_L68	32	55
hVk5-2_L69	50	56
hVk5-2_L70	30, 32	57
hVk5-2_L71	30, 50	58
hVk5-2_L72	30, 32, 50	59
hVk5-2_L73	92	60

Nucleotide sequences of the constructed expression vectors were confirmed by a method known to those skilled in the art. The expression vectors constructed for the altered light chains were transiently introduced, in combination with an expression vector for the heavy chain CIM_H (SEQ ID NO: 48), into cells of the human fetal kidney cell-derived HEK293H line (Invitrogen) or FreeStyle293 (Invitrogen) to express antibodies. From the obtained culture supernatants, antibodies were purified using the rProtein A SepharoseTM Fast Flow (GE Healthcare) by a method known to those skilled in the art. Absorbance at 280 nm of the purified antibody solutions was measured using a spectrophotometer. Antibody concentrations were calculated from the determined values using an extinction coefficient calculated by the PACE method (Protein Science (1995) 4: 2411-2423).

(14-3) Assessment of the calcium ion-binding activity of antibodies having an Ala substitution in the hVk5-2 sequence

Whether the obtained purified antibodies bind to calcium ion was tested. Specifically, the purified antibodies were dialyzed (EasySEP, TOMY) against 20 mM Tris-HCl/150 mM NaCl/2 mM $CaCl_2$ (pH 7.5) solution or 20 mM Tris-HCl/150 mM NaCl (pH 7.5) solution (in Table 19, indicated as 0 μM calcium ion concentration). DSC measurement was carried out at a rate of temperature increase of 240°C/hr from 20 to 115°C using antibody solutions prepared at a concentration of 0.1 mg/mL by the same solution used for dialysis. Based on the obtained DSC denaturation curves, the intermediate temperature of thermal denaturation (Tm value) was calculated for the Fab domain of each antibody as shown in Table 19. Some antibodies having

substitution of an Asp or Glu residue in the CDR sequence of the hVk5-2 sequence with an Ala residue which cannot be involved in calcium ion binding or chelation were revealed to have an Fab domain whose Tm did not vary by the calcium ion concentration in the antibody solutions. The substitution sites at which Ala substitution did not alter the Tm (positions 32 and 92 (Kabat's numbering)) were demonstrated to be greatly important for the calcium ion-antibody binding.

[Table 19]

LIGHT CHAIN VARIANT NAME	ALTERED POSITION (Kabat's NUMBERING)	CALCIUM ION CONCENTRATION		ΔTm (°C)
		0 μM	2 mM	2 mM-0 μM
hVk5-2_L65	WILDTYPE	71.71	73.69	1.98
hVk5-2_L66	30	71.65	72.83	1.18
hVk5-2_L67	31	71.52	73.30	1.78
hVk5-2_L68	32	73.25	74.03	0.78
hVk5-2_L69	50	72.00	73.97	1.97
hVk5-2_L70	30, 32	73.42	73.60	0.18
hVk5-2_L71	30, 50	71.84	72.57	0.73
hVk5-2_L72	30, 32, 50	75.04	75.17	0.13
hVk5-2_L73	92	75.23	75.04	-0.19

[Example 15] Assessment of the calcium ion-binding activity of antibodies having hVk1 sequence with calcium ion-binding motif

(15-1) Construction of an hVk1 sequence with calcium ion-binding motif, and expression and purification of antibodies

The result described in Example 14 on the calcium-binding activity of the Ala substitute demonstrates that Asp or Glu residues in the CDR sequence of the hVk5-2 sequence were important for calcium binding. Thus, the present inventors assessed whether an antibody can bind to calcium ion when the residues at positions 30, 31, 32, 50, and 92 (Kabat's numbering) alone were introduced into a different germline variable region sequence. Specifically, variant LfVk1_Ca (SEQ ID NO: 61) was constructed by substituting the residues at positions 30, 31, 32, 50, and 92 (Kabat's numbering) in the hVk5-2 sequence for the residues at positions 30, 31, 32, 50, and 92 (Kabat's numbering) in the hVk1 sequence (a human germline sequence). Specifically, it was tested whether antibodies having an hVk1 sequence introduced with only 5 residues from the hVk5-2 sequence can bind to calcium. The variants were produced by the

same method as described in Example 2. The resulting light chain variant LfVk1_Ca and LfVk1 having the light-chain hVk1 sequence (SEQ ID NO: 62) were co-expressed with the heavy chain CIM_H (SEQ ID NO: 48). Antibodies were expressed and purified by the same method as described in Example 14.

(15-2) Assessment of the calcium ion-binding activity of antibodies having a human hVk1 sequence with calcium ion-binding motif

Whether the purified antibody prepared as described above binds to calcium ion was assessed by the method described in Example 4. The result is shown in Table 20. The Tm value of the Fab domain of the antibody having LfVk1 with an hVk1 sequence did not vary depending on the calcium concentration in the antibody solutions. Meanwhile, Tm of the antibody having the LfVk1_Ca sequence was shifted by 1°C or more upon change in the calcium concentration in the antibody solutions. Thus, it was shown that the antibody having LfVk1_Ca binds to calcium. The result described above demonstrates that the entire CDR sequence of hVk5-2 is not required, while the residues introduced for construction of the LfVk1_Ca sequence alone are sufficient for calcium ion binding.

[Table 20]

LIGHT CHAIN VARIANT	CALCIUM ION CONCENTRATION		ΔTm(°C)
	3 μM	2 mM	2 mM-3 μM
LfVk1	83.18	83.81	0.63
LfVk1_Ca	79.83	82.24	2.41

(15-3) Construction, expression, and purification of degradation-resistant LfVk1_Ca sequence

As described in (15-2) of Example 15, variant LfVk1_Ca (SEQ ID NO: 61) was constructed to have substitution of residues at positions 30, 31, 32, 50, and 92 (Kabat's numbering) in the hVk5-2 sequence for residues at positions 30, 31, 32, 50, and 92 (Kabat's numbering) in the hVk1 sequence (a human germline sequence). The variant was demonstrated to bind to calcium ion. Thus, one can consider Ca-dependent antibodies (Ca-binding antibodies) having the LfVk1_Ca sequence. However, since the LfVk1_Ca sequence is a novel sequence, its storage stability as pharmaceuticals is unclear. Thus, applicability of the LfVk1_Ca sequence as pharmaceuticals remains to be clarified. In this context, the stability of LfVk1_Ca was assessed by a thermal acceleration test. An antibody having LfVk1_Ca as an L chain was dialyzed against a solution of 20 mM histidine-HCl/150 mM NaCl (pH 6.0) overnight at 4°C. The dialyzed

antibody concentration was adjusted to 0.5 mg/ml, and stored at 5°C or 50°C for three days. After storage, each antibody was subjected to ion-exchange chromatography by the method described in Example 12. The result demonstrated that LfVk1_Ca was significantly degraded during three days of storage at 50°C, as shown in Fig. 17. The LfVk1_Ca sequence has Asp at positions 30, 31, and 32 (Kabat's numbering) and thus its CDR1 sequence contains an Asp-Asp sequence which has been reported to be degraded under acidic conditions (J. Pharm. Biomed. Anal. (2008) 47(1): 23-30). This suggests that amino acids at positions 30, 31, and 32 (Kabat's numbering) are a possible degradation site. Then, to avoid degradation of LfVk1_Ca, variants *LfVk1_Ca1 (SEQ ID NO: 72), LfVk1_Ca2 (SEQ ID NO: 73), and LfVk1_Ca3 (SEQ ID NO:* 74) were constructed to have substitution of Ala (A) residues for the three Asp (D) residues that are possibly sensitive to degradation. Amino acid substitution was carried out by a method known to those skilled in the art using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). DNAs encoding the variants were inserted into animal expression vectors. In combination with an animal expression vector having an insert to express GC H (SEQ ID NO: 102) as the heavy chain, the constructed animal expression vectors carrying DNA inserts for the variants were introduced into animal cells by the method described in Example 14. The antibodies expressed in the animal cells introduced with the vectors were purified by the method described in Example 14.

(15-4) Stability assessment of antibodies having the degradation-resistant LfVk1_Ca sequence

Whether the antibodies prepared as described in (15-3) of Example 15 were more resistant to degradation in solutions at pH 6.0 than the original antibodies having the LfVk1_Ca sequence provided for modification was assessed by comparing the heterogeneity between respective antibodies after thermal acceleration. In the same manner as described above, antibodies were stored at 5°C or 50°C for three days. Each antibody after storage was subjected to ion-exchange chromatography using the method described in Example 12. As shown in Fig. 17, the analysis result demonstrates that LfVk1_Ca1 with an alteration at position 30 (Kabat's numbering) was less heterogeneous and much more resistant to degradation from thermal acceleration than the original LfVk1_Ca sequence. Specifically, it was demonstrated that degradation occurred at the Asp (D) residue of position 30 in the LfVk1_Ca sequence but it could be prevented by amino acid alteration.

(15-5) Construction of a light chain LfVk1_Ca sequence resistant to degradation at the Asp residue of position 30, and expression and purification of antibodies

The result described in (15-4) of Example 15 on the degradation resistance of the Ala-substituted form demonstrates that under acidic conditions the LfVk1_Ca sequence was

degraded at the Asp (D) residue of position 30 (Kabat's numbering) in its CDR sequence and the degradation could be prevented in the case substitution of a different amino acid (in (15-4), by substituting an Ala (A) residue) for the Asp (D) residue at position 30 (Kabat's numbering). Then, the present inventors tested whether even a sequence with a substitution of Ser (S), a residue capable of chelating calcium ion, for the residue at position 30 (Kabat's numbering) (referred to as LfVk1_Ca6; SEQ ID NO: 75) was resistant to degradation while maintaining the calcium-binding activity. Variants were prepared by the same method as described in Example 14. The altered light chains LfVk1_Ca6 and LfVk1_Ca sequences were expressed in combination with a heavy chain GC_H (SEQ ID NO: 102). Antibodies were expressed and purified by the same method as described in Example 14.

(15-6) Assessment of a light chain LfVk1_Ca sequence resistant to degradation at Asp residue at position 30

Purified antibodies prepared as described above were assessed for their storage stability under acidic conditions by the method described in (15-4) of Example 15. The result demonstrates that antibodies having the LfVk1_Ca6 sequence are more resistant to degradation than those having the original LfVk1_Ca sequence, as shown in Fig. 18.

Then, whether antibodies having the LfVk1_Ca sequence and antibodies having the LfVk1_Ca6 sequence bind to calcium ion was tested by the method described in Example 15. The result is shown in Table 21. The Tm values of the Fab domains of antibodies having LfVk1_Ca sequence and antibodies having the degradation-resistant LfVk1_Ca6 sequence were shifted by 1°C or more upon change in the calcium concentration in antibody solutions.

[Table 21]

LIGHT CHAIN VARIANT	CALCIUM ION CONCENTRATION		ΔTm (°C)
	3 μM	2 mM	2 mM-3 μM
LfVk1_Ca	78.45	80.06	1.61
LfVk1_Ca6	78.44	79.74	1.30

Taking the stability into consideration, the result described above demonstrates that it is important for the calcium ion binding of antibodies that the amino acid at position 30 was an amino acid capable of interacting with calcium ion (Asn, Glu, Gln, Ser, Thr, His, Tyr, etc.) other than Asp, and all or some of the amino acids at positions 31, 32, 50, and 92 (Kabat's numbering) in the sequence were the same as hVk5-2 or amino acids capable of interacting with calcium (Asp, Asn, Glu, Gln, Ser, Thr, His, Tyr, etc.). For example, when a synthetic library is

constructed to have such a motif, calcium-dependent binding antibodies can be efficiently isolated from the library.

[Example 16] NMR assessment of the calcium ion-binding activity of antibodies having the human hVk1 sequence with a calcium ion-binding motif

(16-1) Expression and purification of antibodies

An antibody having LfVk1_Ca and an antibody having LfVk1 were expressed and purified for NMR measurements. Specifically, animal expression plasmids for an antibody having LfVk1_Ca were constructed to be capable of expressing its heavy chain (SEQ ID NO: 13) and light chain (SEQ ID NO: 61), and they were introduced transiently into animal cells. Furthermore, animal expression plasmids for an antibody having LfVk1 were constructed to be capable of expressing its heavy chain (SEQ ID NO: 13) and light chain (SEQ ID NO: 62), and they were introduced transiently into animal cells. Labeled amino acids were added to 100 ml of cell suspensions prepared by suspending human fetal kidney cell-derived FreeStyle 293-F (Invitrogen) at a final cell density of 1×10^6 cells/ml in the FreeStyle 293 Expression Medium (Invitrogen). Specifically, a solution of L-aspartic acid-$^{13}C_4$,^{15}N (10 mg), L-glutamic acid-$^{13}C_5$,^{15}N (2.5 mg), L-glutamine-$^{13}C_5$,$^{15}N_2$ (60 mg), L-asparagine-$^{13}C_4$,$^{15}N_2 \cdot H_2O$ (2.5 mg), and β-chloro-L-alanine (6 mg) in 10 ml of water was filtered through a 0.22-μm filter and added to prepare Asp/Glu/Gln/Asn-labeled antibodies. Meanwhile, a solution of L-leucine-^{15}N (30 mg) and β-chloro-L-alanine (6 mg) in 10 ml of water was filtered through a 0.22-μm filter and added to prepare Leu-labeled antibodies. Constructed plasmids were introduced into cells by the lipofection method. Cells introduced with the plasmids were cultured for five days in a CO_2 incubator (37°C, 8% CO_2, 90 rpm). From the culture supernatants prepared as described above, antibodies were purified using the rProtein A Sepharose™ Fast Flow (Amersham Biosciences) by a method known to those skilled in the art. Absorbance at 280 nm of purified antibody solutions was measured using a spectrophotometer. Antibody concentrations were calculated from the determined values using an extinction coefficient calculated by the PACE method (Protein Science (1995) 4: 2411-2423).

(16-2) Preparation of Fab fragment

Each antibody was concentrated to 8.2 to 11.8 mg/ml using an ultrafilter with a molecular weight cut off of 30,000 MWCO. The antibodies were diluted to 8 mg/ml using 50 mM acetic acid/125 mM Tris buffer (pH 6.8) containing 1 mM L-cysteine and 2 mM EDTA to prepare samples. A 1/240 amount of papain (Roche Applied Science) was added to each antibody. After stirring, the samples were incubated at 37°C for one hour. After incubation, each sample was loaded onto a 1-ml HiTrap NHS-activated HP (GE Healthcare) immobilized with

Gly-Gly-Tyr-Arg peptide (Sigma) and equilibrated with 50 mM acetic acid/125 mM Tris buffer (pH 6.8), downstream of which a 1-ml HiTrap MabSelect Sure Protein A column (GE Healthcare) was connected in tandem. Purified Fab fragment fractions were obtained by removing Fc fragment and undigested antibodies by the downstream Protein A column while removing activated papain by the upstream Gly-Gly-Tyr-Arg peptide. Cysteine protease inhibitor E64 (Sigma) was added at 10 μM to the Fab fractions to prevent the activation of inactive papain in the Fab fractions. All the column operations described above were carried out at room temperature from 20 to 25°C.

(16-3) Preparation of Fab fragments of antibodies LfVk1_Ca and LfVk1 as NMR samples

Antibody solutions were concentrated to 0.5 ml by centrifugation using ultrafiltration device Vivaspin (Sartorius) with MWCO 5,000. Then, a diafiltration cup was placed in the ultrafiltration device described above, and the buffer was changed with NMR buffer: 5 mM d-BisTris/20 mM NaCl/0.001%(w/v) NaN_3/5%(v/v) ^2H_2O (pH 7.0) (the pH was adjusted using NaOH and HCl) (via three cycles of: addition of 5 ml of the above-described buffer to the diafiltration cup, followed by concentration to 0.5 ml by centrifugation). The antibody solutions were ultimately concentrated to 0.25 ml. Finally, the ultrafiltration device was washed with NMR buffer, and the buffer was combined with the concentrate. This yielded 420 μl and 270 μl of antibody solutions for antibody LfVk1_Ca and antibody LfVk1, respectively. At this stage, the pH of the solutions was again confirmed, and the pH was adjusted to pH 7.0 using NaOH and HCl if needed. The absorbance at 280 nm was measured using an UV spectrophotometer Nanodrop (Thermo Fisher Scientific) and concentrations of the Fab fragments were determined with molar extinction coefficient at 280 nm = 70,000 $M^{-1}cm^{-1}$. The concentrations of Leu-labeled antibodies LfVk1_Ca and LfVk1 were 0.12 mM, while the concentrations of Asp-, Glu-, Asn-, and Gln-labeled antibodies LfVk1_Ca and LfVk1 were 0.24 mM. Of the above-described samples, antibody LfVk1_Ca was filled in a 5 mm-diameter NMR sample tube (shigemi) and antibody LfVk1 was filled in a 5 mm-diameter symmetrical micro sample tube (shigemi) for aqueous solution using a Pasteur pipette. In Ca^{2+} titration experiments for antibody LfVk1_Ca, $CaCl_2$ solutions were added to antibody solutions in succession so that Ca^{2+} was 1, 2, 5, 10, or 20 molar equivalents to antibody. The $CaCl_2$ solutions added were prepared at 10, 20, 50, and 100 mM $CaCl_2$ using NMR buffer. Required volumes of $CaCl_2$ solutions were added directly to antibody solutions in the NMR sample tubes using a microsyringe (ITO), which was custom-tailored by extending the syringe portion of a ready-made product, so that the loading volume ranges from 3 to 10 μl. After stirring with a vortex mixer, the sample tubes were centrifuged using a manual centrifuge (Shimadzu).

(16-4) NMR measurement to observe amide group signals from the Fab fragments of antibodies LfVk1_Ca and LfVk1_Ca

NMR measurements were carried out using the NMR spectrometer DRX750 (Bruker Biospin) installed with TCI CryoProbe. The temperature was set at 307K (GasFlow 535 L/h). ^{1}H-^{15}N HSQC was used for observing amide group signals in NMR measurements. The measurement method was conducted by simultaneous ^{13}C decoupling of α and carbonyl carbons and subtraction of solvent water signals during the ^{15}N evolution period using ^{1}H-^{15}N FHSQC with a 3-9-19 pulse train. A standard program provided by the manufacturer (Bruker Biospin) was used as a pulse control scheme. The conditions of NMR measurement were as follows. Spectral width: 12019Hz (f2), 1976 Hz (f1); the number of data points: 2048 (f2), 128 (f1). The data were processed using Topspin 3.0 (Bruker Biospin) in the following manner. A shifted square sine (QSINE) window function in both f2 and f1, and zero-filling to double the data size were applied prior to Fourier transformation. The chemical shifts of signals were calculated using an NMR analysis software Sparky (UCSF).

(16-5) NMR signal assignment of main chain amide groups

80% of the NMR signals from the main chain amide groups of the Fab fragment of tocilizumab (heavy chain SEQ ID NO: 13; light chain SEQ ID NO: 14) were assigned previously (data not disclosed). The amino acid sequence of the Fab fragment of antibody LfVk1_Ca is the same as that of the Fab fragment of tocilizumab, except some portions of light chain CDR1, CDR2, CDR3 and the amino acid residues at positions 73 and 83 in the light chain. Amino acid sequences shared by the two antibodies give NMR signals that exhibit the same or similar chemical shifts. Because of this, the assignment information on tocilizumab was applicable in such amino acid sequences. For Leu-labeled samples, assignments revealed to be applicable include: 11, (33), (46), (47), (54), (78), 125, 135, 136, 154, 175, 179, 181, and 201 in the light chain, and 18, 46, 64, 71, 81, 83, 114, 144, 147, 165, 176, 181, 184, and 195 in the heavy chain. In the above, numbers without parenthesis represent residue numbers at which the assignments are applicable because the chemical shifts are shared by tocilizumab; numbers in parentheses represent residue numbers at which the assignments are applicable because the chemical shifts are similar to those of tocilizumab and there are no other signals giving similar chemical shifts. Meanwhile, for the Asp-, Glu-, Asn-, Gln-labeled samples, four signals were newly observed in LfVk1_Ca when the spectra were compared between antibodies LfVk1_Ca and LfVk1. These were assumed to be assignable to four of the five residues, Asp30, Asp31, Asp32, Asp92, and Glu50, among Asp, Glu, Asn, and Gln residues in the light chain where the sequence introduced as a Ca^{2+}-binding motif is different between the two antibodies.

(16-6) Identification of Ca^{2+} binding site in antibody LfVk1_Ca

Signals with different chemical shift were extracted by comparing ^{1}H-^{15}N HSQC spectra of the Fab fragment of antibody LfVk1_Ca between in the presence and absence of 20 molar equivalents of Ca^{2+}. The result on the Leu-labeled samples showed that only Leu33, but no other Leu residues, in the light chain is involved in the binding. In addition, with the Asp-, Glu-, Asn-, Gln-labeled samples, four of the five residues, Asp30, Asp31, Asp32, Asp92, and Glu50, in the light chain were revealed to be involved in the binding, and all but except one of the other Asp, Glu, Asn, and Gln residues were not responsible for the binding. The finding described above demonstrates that in the amino acid sequence introduced as a Ca^{2+}-binding motif, some amino acids of at least light chain CDR1 and of both or either of light chain CDR2 and CDR3 were involved in the Ca^{2+} binding. This is consistent with the finding described in Example 15 that it is important for the calcium ion binding that amino acids at four positions among positions 30, 31, 32, 50, and 92 (Kabat's numbering) are identical to those in the hVk5-2 sequence.

(16-7) Calculation of Ca^{2+} dissociation constant by titration experiment

Based on ^{1}H-^{15}N HSQC spectra at Ca^{2+} concentrations of 0, 1, 2, 5, 10, or 20 molar equivalents to the Fab fragment of antibody LfVk1_Ca, a graph was plotted with the molar equivalent of Ca^{2+} in the horizontal axis and with ^{1}H or ^{15}N chemical shifts of the signal for light chain Leu33 identified as the binding site in the vertical axis. Using the function represented by formula 2 shown below, data fitting was performed with graphing software Gnuplot.

[Formula 2]

$$f(x) = s * [1-0.5/a * \{(a * x + a + Kd) - ((a * x + a + Kd)^2 - 4 * x * a^2)^{0.5}\} + t * [0.5/a * \{(a * x + a + Kd) - ((a * x + a + Kd)^2 - 4 * x * a^2)^{0.5}\}$$

In the function represented by formula 2, "s" and "t" represent the chemical shift [ppm] for the Ca^{2+}-unbound state and an estimated chemical shift [ppm] for the Ca^{2+}-bound, saturated state, respectively; "a" represents the concentration of the antibody Fab fragment [M]; "Kd" represents the dissociation constant; and "x" represents the molar equivalents of Ca^{2+} added to the antibody Fab fragment. In the data fitting, s, t, and Kd were fitting parameters. As a result, based on ^{1}H and ^{15}N chemical shifts, Kd was estimated as follows: Kd = 7.1 x 10^{-5} [M] and Kd = 5.9 x 10^{-5} [M], respectively.

[Example 17] Assessment of variant sequence hVk5-2 for calcium binding

Vk5-2 variant 1 (SEQ ID NO: 63) and Vk5-2 variant 2 (SEQ ID NO: 64) were obtained in addition to Vk5-2 (SEQ ID NO: 41), all of which are classified as Vk5-2. These variants were

assessed for their calcium binding. DNA fragments for Vk5-2, Vk5-2 variant 1, and Vk5-2 variant 2 were each inserted into animal cell expression vectors. The nucleotide sequences of the constructed expression vectors were determined by a method known to those skilled in the art. By the method described in Example 13, the animal cell expression vectors inserted with DNA fragments for Vk5-2, Vk5-2 variant 1, and Vk5-2 variant 2 were introduced, in combination with animal expression vector carrying an insert to express CIM_H (SEQ ID NO: 48) as a heavy chain, into animal cells and antibodies were purified. The purified antibodies were assessed for their calcium ion-binding activity. The purified antibodies were dialyzed (EasySEP, TOMY) against 20 mM Tris-HCl/150 mM NaCl (pH 7.5) (in Table 22, indicated as 0 mM calcium ion concentration) or 20 mM Tris-HCl/150 mM NaCl/2 mM $CaCl_2$ (pH 7.5). DSC measurement was carried out at a rate of temperature increase of 240°C/hr from 20 to 115°C using antibody solutions prepared at a concentration of 0.1 mg/mL by the same solution as used for dialysis. Based on the obtained DSC denaturation curves, the intermediate temperature of thermal denaturation (Tm value) was calculated for the Fab domain of each antibody. The Tm values are shown in Table 22.

[Table 22]

LIGHT CHAIN	CALCIUM ION CONCENTRATION		ΔTm (°C)
	0 mM	2 mM	2 mM-0mM
Vk5-2	71.65	74.38	2.73
Vk5-2 VARIANT 1	65.75	72.24	6.49
Vk5-2 VARIANT 2	66.46	72.24	5.78

The result showed that the Tm value for the Fab domains of antibodies having the sequence of Vk5-2, Vk5-2 variant 1, or Vk5-2 variant 2 varied depending on the calcium ion concentration in solutions containing antibodies having the Fab domains. This demonstrates that antibodies having a sequence classified as Vk5-2 bind to calcium ion.

[Example 18] Antibodies that bind to human CD4 in a calcium-dependent manner

(18-1) Preparation of soluble human CD4

Soluble human CD4 was prepared as follows. A DNA sequence encoding a sequence (SEQ ID NO: 76) in which Myc tag is attached to the amino acid sequence of human CD4 that lacks the transmembrane region was inserted into an animal cell expression vector. The sequence of the constructed recombinant human CD4 was confirmed by a method known to those skilled in the art.

(18-2) Expression and purification of antibodies that bind to soluble human CD4

TNX355-IgG1 (heavy chain SEQ ID NO: 77; light chain SEQ ID NO: 78) and Q425 (heavy chain SEQ ID NO: 79; light chain SEQ ID NO: 80) are anti-human CD4 antibodies. Furthermore, Q425L9 (heavy chain SEQ ID NO: 81; light chain SEQ ID NO: 82) is an L chain variant from Q425. DNA sequences encoding the amino acids of TNX355-IgG1 (heavy chain SEQ ID NO: 77; light chain SEQ ID NO: 78), Q425 (heavy chain SEQ ID NO: 79; light chain SEQ ID NO: 80), and Q425L9 (heavy chain SEQ ID NO: 81; light chain SEQ ID NO: 82) were inserted into animal cell expression plasmids. Antibodies were expressed by the following method. Cells of human fetal kidney cell-derived FreeStyle 293-F (Invitrogen) were suspended in FreeStyle 293 Expression Medium (Invitrogen), and plated at a cell density of 1.33×10^6 cells/ml (3 ml) into each well of a 6-well plate. The prepared plasmids were introduced into cells by a lipofection method. The cells were cultured for four days in a CO_2 incubator (37°C, 8% CO_2, 90 rpm). From the culture supernatants prepared as described above, antibodies were purified using the rProtein A Sepharose™ Fast Flow (Amersham Biosciences) by a method known to those skilled in the art. Absorbance at 280 nm of purified antibody solutions was measured using a spectrophotometer. Antibody concentrations were calculated from the determined values using an extinction coefficient calculated by the PACE method (Protein Science (1995) 4: 2411-2423).

(18-3) Assessment of prepared antibodies for calcium-dependent binding activity to human CD4

The prepared antibodies were assessed for their calcium-dependent binding activity to soluble human CD4 using Biacore T100 (GE Healthcare). The high calcium ion concentration used was 1.2 mM, while the low calcium ion concentration was 3 μM. Soluble human CD4 (prepared as described in 18-1) was used as antigen. An adequate amount of protein G (Invitrogen) was immobilized onto the Sensor chip CM4 (GE Healthcare) by the amine coupling method, and then antibodies of interest were allowed to capture. 10 mmol/l ACES, 150 mmol/l NaCl, 0.05% (w/v) Tween20, 1.2 mmol/l $CaCl_2$ (pH 7.4 or pH 6.0) containing 1.2 mmol/l or 3 μmol/l $CaCl_2$ was used as a running buffer. All measurements were carried out at 37°C. Human CD4 was diluted using the respective buffers. Antibody sensorgrams are shown in Fig. 19. As shown in Fig. 19, the shape of sensorgram of antibody TNX355-IgG1 did not change even when the running buffer condition was changed. This demonstrates that TNX355-IgG1 is a common antibody that does not show calcium-dependent binding activity to human CD4. Meanwhile, for both antibodies Q425 and Q425L9, the amount of antigen binding was smaller at a calcium ion concentration of 3 μM (low calcium ion concentration) than at 1.2 mM (high calcium ion concentration), and thus they exhibited Ca-dependent binding activity. In particular, no binding

phase was observed for antibody Q425L9 at a calcium ion concentration of 3 μM even at an analyte (soluble human CD4) concentration of 200 nM. Specifically, Q425 and Q425L9 were demonstrated to be calcium-dependent binding antibodies that bind to human CD4 in a calcium-dependent manner.

[Example 19] Assessment of Ca-dependent binding antibodies for their effect on antigen retention in plasma using normal mice

(19-1) *In vivo* assay using normal mice

Q425 and Q425L9 prepared as described in Example 18 are antibodies that bind to soluble human CD4 in a calcium-dependent manner. As already described in Examples 5 and 6, regarding IL6R, it has been demonstrated that when administered in combination with an antigen, an antibody having the property of binding to an antigen in a calcium-dependent manner has a property to accelerate antigen elimination as compared to when an antibody that binds to an antigen in a calcium-independent manner is administered in combination with an antigen. However, whether antibodies against other antigens also have the property to accelerate antigen elimination remain to be clarified.

Then, soluble human CD4 (prepared as described in Example 18) was administered alone or in combination with an anti-human CD4 antibody to normal mice (C57BL/6J mouse; Charles River Japan). The mice were assessed for *in vivo* kinetics of soluble human CD4 and anti-human CD4 antibody after administration. A solution of soluble human CD4 (50 μg/ml) or a mixed solution of soluble human CD4 and an anti-human CD4 antibody was administrated once at 10 ml/kg to the caudal vein. Anti-human CD4 antibodies used were TNX355-IgG1, Q425-IgG1, and Q425L9-IgG1 described above.

The concentration of soluble human CD4 in the mixed solution was 50 μg/ml. Meanwhile, the concentrations of anti-human CD4 antibodies varied depending on the antibody: 0.264 mg/ml for TNX355-IgG1; 0.197 mg/ml for Q425-IgG1; and 2.594 mg/ml for Q425L9-IgG1. In this case, the anti-human CD4 antibodies were present in an excess amount as compared to soluble human CD4, and soluble human CD4 was assumed to mostly bind to the antibodies. In the group administered with soluble human CD4 alone, blood was collected 2 minutes, 5 minutes, 15 minutes, 30 minutes, one hour, and two hours after administration. In the group administered with soluble human CD4 in combination with TNX355-IgG1 without calcium-dependent antigen-binding activity, blood was collected 5 minutes, 2 hours, 7 hours, 1 day, 3 days, 7 days, 14 days, and 28 days after administration. In the group administered with soluble human CD4 in combination with Q425-IgG1 or Q425L9-IgG1 having calcium-dependent antigen-binding activity, blood was collected 5 minutes, 30 minutes, 2 hours, 7 hours, 1 day, 3 days, 8 days, 14 days, and 28 days after administration. Immediately after collection,

the blood was centrifuged at 4°C and 12,000 rpm for 15 minutes to isolate plasma. The isolated plasma was stored in a freezer at -20°C or below before measurements.

(19-2) Determination of plasma anti-human CD4 antibody concentration in normal mice by ELISA

Anti-human CD4 antibody concentrations in mouse plasma were determined by ELISA. First, Anti-Human IgG (γ-chain specific) F(ab')2 Fragment of Antibody (SIGMA) was aliquoted into Nunc-Immuno Plate, MaxiSorp (Nalge nunc International). The plate was allowed to stand overnight at 4°C to prepare an anti-human IgG antibody-immobilized plate. Standard samples were prepared at concentrations of 0.64, 0.32, 0.16, 0.08, 0.04, 0.02, and 0.01 μg/ml in plasma. Mouse plasma assay samples were prepared by diluting 100 times or more. The samples were aliquoted into the anti-human IgG antibody-immobilized plate. The plate was incubated at 25°C for one hour. After incubation, the samples were reacted with biotinylated anti-human IL-6 R antibody (R&D) at 25°C for one hour, and then with Streptavidin-*PolyHRP80* (Stereospecific Detection Technologies) at 25°C for 0.5 hour. Chromogenic reaction was carried out using TMB One Component HRP Microwell Substrate (BioFX Laboratories) as a substrate. After the reaction was terminated with 1N sulfuric acid (Showa Chemical), the absorbance at 450 nm was measured using a microplate reader. Using analysis software SOFTmax PRO (Molecular Devices), the concentrations in mouse plasma were calculated based on the absorbance from the standard curve.

A time course of plasma concentrations of antibodies TNX355-IgG1, Q425-IgG1, and Q425L9-IgG1 determined by the above-described method after intravenous administration to normal mice is shown in Fig. 20.

(19-3) Determination of plasma concentrations of soluble human CD4 by an electrochemical luminescence method

Soluble human CD4 concentrations in mouse plasma were determined by ELISA.

For the group administered with sCD4 alone and the group administered in combination with Q425 or Q425_L9, TNX was aliquoted into Nunc-Immuno Plate, MaxiSorp (Nalge nunc International). The plate was left overnight at 4°C to prepare a TNX-immobilized plate. Standard samples were prepared at plasma concentrations of 10, 5, 2.5, 1.25, 0.625, 0.3125, and 0.156 μg/ml. Mouse plasma assay samples were prepared by diluting 100 times or more. The samples were prepared using a buffer containing 10 mM EDTA, and aliquoted into the TNX-immobilized plate. After three hours of incubation at 25°C, the samples were reacted with anti-c-myc-HRP (Miltenyi Biotech) at 25°C for one hour. Chromogenic reaction was carried out using the TMB One Component HRP Microwell Substrate (BioFX Laboratories) as a substrate.

After the reaction was terminated with 1N sulfuric acid (Showa Chemical), the absorbance at 450 nm was measured using a microplate reader. Using the analysis software SOFTmax PRO (Molecular Devices), the concentrations in mouse plasma were calculated based on the absorbance from the standard curve.

In the group administered in combination with TNX, Q425 was aliquoted into Nunc-Immuno Plate, MaxiSorp (Nalge nunc International). The plate was left overnight at 4°C to prepare a Q425-immobilized plate. Standard samples were prepared at plasma concentrations of 20, 10, 5, 2.5, 1.25, 0.625, and 0.3125 μg/ml. Mouse plasma assay samples were prepared by diluting 100 times or more. The samples were prepared using a buffer containing 2 mM Ca^{2+}, and aliquoted into the TNX-immobilized plate. After three hours of incubation at 25°C, the samples were reacted with Anti-c-myc-HRP (Miltenyi Biotech) at 25°C for one hour. Chromogenic reaction was carried out using the TMB One Component HRP Microwell Substrate (BioFX Laboratories) as a substrate. After the reaction was terminated with 1N sulfuric acid (Showa Chemical), the absorbance at 450 nm was measured using a microplate reader. Using the analysis software SOFTmax PRO (Molecular Devices), the concentrations in mouse plasma were calculated based on the absorbance from the standard curve.

A time course of plasma concentrations of soluble human CD4 determined by the above-described method after intravenous administration to normal mice is shown in Fig. 21.

The result showed that soluble human CD4 when administered alone was eliminated very rapidly. Meanwhile, the elimination of soluble human CD4 was greatly retarded when administered in combination with TNX355-IgG1, a common antibody without Ca-dependent binding activity to soluble human CD4. In contrast, the elimination of soluble human CD4 was significantly accelerated when administered in combination with Q425-IgG1 or Q425L9-IgG1 having Ca-dependent binding activity to soluble human CD4. The elimination of soluble human CD4 could be accelerated when administered in combination with Q425-IgG1 or Q425L9-IgG1 as compared to when administered in combination with TNX355-IgG1. This finding demonstrates that not only for IL-6R but also for human CD4, antigen elimination from plasma can be achieved with a calcium-dependent binding antibody.

[Example 20] Antibodies that bind to human IgA in a calcium-dependent manner

(20-1) Preparation of human IgA (hIgA)

An antigen, recombinant human IgA (hereinafter abbreviated as hIgA), was prepared as follows. hIgA comprising H(WT)-IgA1 (SEQ ID NO: 83) and L(WT) (SEQ ID NO: 14) was expressed, and purified by ion-exchange chromatography and gel filtration chromatography using a method known to those skilled in the art.

(20-2) Expression and purification of antibodies that bind to human IgA

GA1-IgG1 (heavy chain SEQ ID NO: 84; light chain SEQ ID NO: 85), GA2-IgG1 (heavy chain SEQ ID NO: 86; light chain SEQ ID NO: 87), GA3-IgG1 (heavy chain SEQ ID NO: 88; light chain SEQ ID NO: 89), and GA4-IgG1 (heavy chain SEQ ID NO: 90; light chain SEQ ID NO: 91) are antibodies that bind to human IgA. Then, for the purpose of further enhancing antigen (hIgA) elimination from plasma, in a similar way as described in Examples 6 and 7, GA2-N434W (heavy chain SEQ ID NO: 92; light chain SEQ ID NO: 87) was constructed by introducing amino acid substitution N434W into GA2-IgG1 to strengthen the binding to mouse FcRn at pH 7.4. DNA sequences encoding GA1-IgG1 (heavy chain SEQ ID NO: 84; light chain SEQ ID NO: 85), GA2-IgG1 (heavy chain SEQ ID NO: 86; light chain SEQ ID NO: 87), GA3-IgG1 (heavy chain SEQ ID NO: 88; light chain SEQ ID NO: 89), GA4-IgG1 (heavy chain SEQ ID NO: 90; light chain SEQ ID NO: 91), and GA2-N434W (heavy chain SEQ ID NO: 92; light chain SEQ ID NO: 87) were inserted into animal expression plasmids by a method known to those skilled in the art. Antibodies were expressed by the following method. Cells of human fetal kidney cell-derived FreeStyle 293-F (Invitrogen) were suspended in the FreeStyle 293 Expression Medium (Invitrogen), and plated at a cell density of 1.33×10^6 cells/ml (3 ml) into each well of a 6-well plate. The constructed plasmids were introduced into cells by a lipofection method. The cells were cultured for four days in a CO_2 incubator (37°C, 8% CO_2, 90 rpm). From the prepared culture supernatants, antibodies were purified using the rProtein A Sepharose™ Fast Flow (Amersham Biosciences) by a method known to those skilled in the art. The concentrations of purified antibodies were determined by measuring absorbance at 280 nm using a spectrophotometer. Antibody concentrations were calculated from the determined values using an extinction coefficient calculated by the PACE method (Protein Science (1995) 4: 2411-2423).

(20-3) Assessment of prepared antibodies for Ca-dependent human IgA-binding activity

Using Biacore T200 (GE Healthcare), the obtained antibodies were assessed for their binding activity to human IgA (dissociation constant K_D (M)). The measurement was carried out using as a running buffer 0.05% tween20, 20 mmol/l ACES, 150 mmol/l NaCl (pH 7.4 or pH 5.8) containing 3 μM or 1.2 mM $CaCl_2$, or 0.05% tween20, 20 mmol/l ACES, 150 mmol/l NaCl (pH 8.0) containing 0.1 μM or 10 mM $CaCl_2$.

After an adequate amount of recombinant Protein A/G (Thermo Scientific) was immobilized onto the Sensor chip CM5 (GE Healthcare) by an amino coupling method, antibodies were allowed to bind onto the sensor chip. An appropriate concentration of hIgA (described in (20-1)) was injected as an analyte to interact with antibodies on the sensor chip. Then, the sensor chip was regenerated by injecting 10 mmol/l glycine-HCl, pH 1.5. The

measurement was carried out at 37°C. From the assay result, the dissociation constant K_D (M) was calculated based on curve-fitting analysis and equilibrium constant analysis using Biacore T200 Evaluation Software (GE Healthcare). The result is shown in Table 23. The obtained sensorgram is shown in Fig. 22. GA2-IgG1, GA3-IgG1, and GA4-IgG1 were demonstrated to bind to human IgA strongly at a Ca^{2+} concentration of 1.2 mM and weakly at a Ca^{2+} concentration of 3 μM.

[Table 23]

ANTIBODY NAME	CONDITION	Fit	ka	kd	KD
GA1-IgG1	pH8.0, 10 mM Ca	1:1binding model	1.10E+06	2.40E-01	2.20E-07
	pH8.0, 0.1 μM Ca	1:1binding model	1.20E+06	1.20E-01	1.00E-07
	pH7.4, 1.2 mM Ca	1:1binding model	5.70E+05	8.40E-02	1.50E-07
	pH7.4, 3 μM Ca	1:1binding model	6.40E+05	1.20E-01	1.90E-07
	pH5.8, 1.2 mM Ca	1:1binding model	6.80E+05	9.90E-02	1.40E-07
	pH5.8, 3 μM Ca	1:1binding model	7.10E+05	1.10E-01	1.50E-07
GA2-IgG1	pH7.4, 1.2 mM Ca	1:1binding model	4.00E+05	1.60E-02	3.90E-08
	pH7.4, 3 μM Ca	Steady State Affinity	-	-	6.70E-06
	pH5.8, 1.2 mM Ca	Steady State Affinity	-	-	4.00E-06
	pH5.8, 3 μM Ca	Steady State Affinity	-	-	5.00E-06
GA3-IgG1	pH7.4, 1.2 mM Ca	1:1binding model	4.30E+05	3.30E-02	7.90E-08
	pH7.4, 3 μM Ca	Steady State Affinity	-	-	-
	pH5.8, 1.2 mM Ca	1:1binding model	4.40E+05	3.50E-02	8.10E-08
	pH5.8, 3 μM Ca	Steady State Affinity	-	-	1.10E-06
GA4-IgG1	pH7.4, 1.2 mM Ca	Steady State Affinity	-	-	4.20E-07
	pH7.4, 3 μM Ca	Steady State Affinity	-	-	8.90E-07
	pH5.8, 1.2 mM Ca	Steady State Affinity	-	-	1.10E-06
	pH5.8, 3 μM Ca	Steady State Affinity	-	-	1.50E-06

[Example 21] Assessment of the effect of Ca-dependent human IgA-binding antibodies on antigen retention in plasma using normal mice

(21-1) *In vivo* assay using normal mice

Human IgA (human IgA: prepared as described in Example 20) was administered alone or in combination with an anti-human IgA antibody to normal mice (C57BL/6J mouse; Charles River Japan). The mice were assessed for *in vivo* kinetics of human IgA and anti-human IgA antibody after administration. A human IgA solution (80 μg/ml) or a mixed solution of human IgA and anti-human IgA antibody was administered once at 10 ml/kg to the caudal vein. Anti-human IgA antibodies used were GA1-IgG1, GA2-IgG1, GA3-IgG1, and GA2-N434W

described above.

The concentration of human IgA in the mixed solution was 80 μg/ml. Meanwhile, the concentrations of anti-human IgA antibodies vary depending on the affinity for hIgA: 100 mg/ml for GA1-IgG1; 28.9 mg/ml for GA2-IgG1; 53.8 mg/ml for GA3-IgG1; and 1 mg/ml for GA2-N434W. In this case, the anti-human IgA antibodies were present in an excess amount as compared to human IgA, and human IgA was assumed to mostly bind to the antibodies. Blood was collected 5 minutes, 7 hours, 1 day, 2 days, 3 days, and 7 days after administration. Immediately after the collection, the blood was centrifuged at 4°C and 12,000 rpm for 15 minutes to isolate plasma. The isolated plasma was stored in a freezer at -20°C or below before measurements.

(21-2) Determination of plasma concentration of anti-human IgA antibody in normal mice by ELISA

Anti-human IgA antibody concentrations in mouse plasma were determined by ELISA. First, Anti-Human IgG (γ-chain specific) F(ab')2 Fragment of Antibody (SIGMA) was aliquoted into Nunc-Immuno Plate, MaxiSorp (Nalge nunc International). The plate was left overnight at 4°C to prepare an anti-human IgG antibody-immobilized plate. Standard samples were prepared at plasma concentrations of 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.01563, and 0.07813 μg/ml. Mouse plasma assay samples were prepared by diluting 100 times or more. The samples were aliquoted into the Anti-Human IgG antibody-immobilized plate. After one hour of incubation at 25°C, the samples were reacted with the Goat Anti-Human IgG (γ chain specific) Biotin (BIOT) Conjugate (Southern Biotechnology Associats Inc.) at 25°C for one hour. Then, the samples were reacted with Streptavidin-PolyHRP80 (Stereospecific Detection Technologies) at 25°C for one hour. Chromogenic reaction was carried out using the TMB One Component HRP Microwell Substrate (BioFX Laboratories) as a substrate. After the reaction was terminated with 1N sulfuric acid (Showa Chemical), the absorbance at 450 nm was measured using a microplate reader. Using the analysis software SOFTmax PRO (Molecular Devices), the concentrations in mouse plasma were calculated based on the absorbance from the standard curve. A time course of plasma concentrations of antibodies GA1-IgG1, GA2-IgG1, GA3-IgG1, and GA2-N434W determined by the above-described method after intravenous administration to normal mice is shown in Fig. 23.

(21-3) Determination of plasma human IgA concentration by ELISA

Human IgA concentrations in mouse plasma were determined by ELISA. First, Goat anti-Human IgA antibody (BETHYL) was aliquoted into a Nunc-Immuno Plate, MaxiSorp (Nalge nunc International). The plate was left at 4°C overnight to prepare an anti-human IgA

antibody-immobilized plate. Standard samples of human IgA were prepared at plasma concentrations of 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125, and 0.00625 μg/ml. Mouse plasma assay samples were prepared by diluting 100 times or more. 200 μl of 500 ng/ml hsIL-6R was added to 100 μl of the standard and plasma samples. The resulting mixtures were allowed to stand at room temperature for one hour, and then aliquoted into the anti-human IgA antibody-immobilized plate and incubated at room temperature for one hour. After incubation, the mixtures were reacted with biotinylated Anti-human IL-6R antibody (R&D) at room temperature for one hour, and then with the Streptavidin-PolyHRP80 (Stereospecific Detection Technologies) at room temperature for one hour. Chromogenic reaction was carried out using the TMB One Component HRP Microwell Substrate (BioFX Laboratories) as a substrate. After the reaction was terminated with 1N sulfuric acid (Showa Chemical), the absorbance at 450 nm was measured using a microplate reader. Using analysis software SOFTmax PRO (Molecular Devices), the concentrations in mouse plasma were calculated based on the absorbance from the standard curve. A time course of plasma concentrations of human IgA determined by the above-described method after intravenous administration to normal mice is shown in Fig. 24.

The result showed that when human IgA was administered in combination with GA1-IgG1, an antibody whose Ca dependency in the human IgA binding is weak (the degree of dependency is low), the elimination of human IgA was retarded as compared to when administered alone. Meanwhile, the elimination of human IgA was significantly accelerated when administered in combination with GA2-IgG1 which exhibits 100 times or more Ca-dependent human IgA-binding activity. The plasma concentration of unbound human IgA was determined from the plasma antibody concentration shown in Fig. 23, the plasma concentration of human IgA shown in Fig. 24, and the KD value of each antibody shown in Table 23. The result is shown in Fig. 25. As shown in Fig. 25, the concentration of unbound antigen (human IgA) in the group administered with GA2-IgG1 or GA3-IgG1 was lower as compared to the concentration of unbound antigen (human IgA) in the GA1-IgG1-administered group. This demonstrates that unbound antigen (human IgA) can be reduced by accelerating antigen elimination using calcium-dependent binding antibodies. Moreover, GA2-N434W that exhibited enhanced FcRn binding at pH 7.4 accelerated antigen elimination more than GA2-IgG1. The antigen was reduced to a level below the detection limit 7 hours after administration.

The finding described above demonstrates that calcium-dependent binding antibodies can accelerate antigen elimination from plasma as compared to common antibodies that bind to an antigen in a pH- or calcium-independent manner. It was revealed that this applies not only to human IL6R described in Example 5 or human CD4 described in Example 19 but also to human IgA. Furthermore, in addition to human IL6R described in Examples 6 and 7, it was demonstrated for human IgA that antigen elimination can be further accelerated by enhancing the

FcRn binding of calcium-dependent binding antibodies at pH 7.4.

As shown in Reference Example 31, Fv4-IgG1, which binds to human IL-6 receptor in a pH-dependent manner, can accelerate the elimination of human IL-6 receptor as compared to H54/L28-IgG1 which binds to human IL-6 receptor in a pH-independent manner; however, Fv4-IgG1 cannot accelerate the elimination as compared to administration of human IL-6 receptor alone. Fv4-IgG1-v1 or Fv4-IgG1-v2 with enhanced FcRn binding activity in the neutral range should be used to accelerate the elimination as compared to administration of human IL-6 receptor alone.

Meanwhile, surprisingly, GA2-IgG1, which binds to human IgA in a Ca-dependent manner, was revealed to accelerate the elimination of human IgA as compared to administration of human IgA alone, although it has the constant region of natural IgG1 whose FcRn binding in the neutral range is not enhanced. The following mechanism is thought to account for what happened in GA2-IgG1.

In the case of monomeric antigens such as human IL-6 receptor, two antigens bind to a divalent antibody. This results in the formation of an antigen/antibody complex consisting of three molecules of antigen and antibody. On the other hand, since human IgA is a dimeric antigen and an antibody is divalent, the antigen/antibody complex between them is likely to form an antigen/antibody complex (immune complex) consisting of four or more molecules of antigen and antibody.

When a common antibody of natural IgG1 type against a multimeric antigen forms a bulky immune complex, the immune complex can bind to FcgR, FcRn, complement receptor, and such with avidity in a multivalent fashion via Fc domain. Thus, the immune complex is internalized into cells expressing such receptors. Meanwhile, a common pH/Ca-independent antibody against a monomeric antigen has insufficient affinity for the natural IgG1 type receptor, and thus the resulting immune complex is internalized into cells with low efficiency. FcRn originally has a role of recycling intracellularly internalized antibodies from the endosome to plasma. However, bulky immune complexes capable of binding to FcRn in a multivalent fashion are known to be transferred from the endosome by FcRn and degraded in the lysosome. Specifically, as shown in Fig. 26, a common antibody against a multimeric antigen, which forms a bulky immune complex, can accelerate the elimination of the antigen; however, the antigen is not dissociated from the antibody in the endosome, and the antibody is also eliminated simultaneously together with the antigen. Therefore, the antigen elimination efficiency per antibody molecule is low. In other words, a common pH/Ca-independent antibody against a monomeric antigen can accelerate antigen elimination; however, the efficiency is assumed to be low.

On the other hand, when a pH/Ca-dependent antibody that has a natula-IgG1-type

constant region against a multimeric antigen forms a bulky immune complex, the immune complex binds to FcgR, FcRn, complement receptor, and such with avidity *via* multivalent Fc region as shown in Fig. 27, and is taken up by cells expressing the receptors. The immune complex dissolves by dissociation of the antigen from the pH/Ca-dependent antibody in the endosome. The antigen cannot bind to FcRn and is transferred to the lysosome for degradation. Meanwhile, the antibody is recycled to plasma by FcRn because it does not form an immune complex.

Specifically, when a pH/Ca-dependent antibody that has a natural-IgG1-type constant region against a multimeric antigen can bind to FcgR, FcRn, complement receptor, and such with avidity by forming a bulky immune complex, only antigen elimination can be selectively and greatly accelerated. The above described phenomenon was assumed to also occur with GA2-IgG1 against human IgA. This was expected to be useful as a method for significantly accelerating the elimination of multimeric antigen without using the amino acid substitution method for enhancing the FcRn binding of natural IgG1 in the neutral range such as shown in Reference Example 31.

In order to achieve the effect described above, an antigen and an antibody form a bulky immune complex and must tightly bind to FcgR/FcRn with avidity, even if the antibody is an IgG1. When the antigen is a dimeric or higher-order polymeric antigen, by screening for pH/Ca-dependent antibodies that form a bulky immune complex and bind to the above-described receptor, the antigen elimination can be accelerated efficiently by using the natural IgG1 constant region without performing any amino acid substitution. In general, it is considered that antigens have to be multimeric (for example, immunoglobulins such as IgA and IgE, and the TNF superfamily such as TNF and CD154) for antibodies and antigens to form bulky immune complexes. Even when an antigen is monomeric, a bulky immune complex can be formed by using a mixture of two or more types of appropriate pH/Ca-dependent antibodies that recognize two or more epitopes in a monomeric antigen. Alternatively, a bulky immune complex can be formed by using an appropriate multispecific pH/Ca-dependent antibody that recognizes two or more epitopes in a monomeric antigen (for example, a bispecific antibody having a natural IgG constant region with the right and left arms recognizing epitopes A and B, respectively, such as shown in Fig. 28). Specifically, if appropriate pH/Ca-dependent antibodies against monomeric antigens can be screened, antigen elimination can be accelerated efficiently by using a mixture of antibodies having a natural IgG1 constant region or a multispecific antibody having a natural IgG1 constant region, without using mutant IgG1 having an amino acid substitution.

[Example 22] Antibodies that bind to human glypican 3 in a calcium-dependent manner

(22-1) Preparation of human glypican 3 (GPC3)

Recombinant human glypican 3 (hereinafter abbreviated as GPC3) which is used as an antigen was prepared by the following procedure. CHO cells constitutively introduced with a plasmid that expresses a sequence to which six histidine residues are linked to the amino acid sequence of human glypican 3 without having the transmembrane domain (SEQ ID NO: 93) were cultured. Then, from the collected culture supernatant, GPC3 was purified by ion-exchange chromatography, followed by His tag-based affinity and gel filtration chromatography.

(22-2) Expression and purification of antibodies that bind to human GPC3

Anti-human glypican 3 antibodies CSCM-01_005 (heavy chain sequence: 94; light chain sequence: 95), CSCM-01_009 (heavy chain sequence: 96; light chain sequence: 97), CSCM-01_015 (heavy chain sequence: 98, light chain sequence: 99), CSCM-01_023 (heavy chain sequence: 100; light chain sequence: 101), and GC-IgG1 (heavy chain sequence: 102; light chain sequence: 103) were each inserted into animal expression plasmids. Antibodies were expressed by the following procedure. Cells of human fetal kidney cell-derived FreeStyle 293-F (Invitrogen) were suspended in the FreeStyle 293 Expression Medium (Invitrogen), and plated at a cell density of 1.33×10^6 cells/ml (3 ml) into each well of a 6-well plate. The prepared plasmids were introduced into cells by a lipofection method. The cells were cultured for four days in a CO_2 incubator (37°C, 8% CO_2, 90 rpm). From the prepared culture supernatants, antibodies were purified using the rProtein A Sepharose™ Fast Flow (Amersham Biosciences) by a method known to those skilled in the art. The concentrations of purified antibodies were determined by measuring absorbance at 280 nm using a spectrophotometer. Antibody concentrations were calculated from the determined values using an extinction coefficient calculated by the PACE method (Protein Science (1995) 4: 2411-2423). Furthermore, antibody GC-IgG1 was purified from culture supernatants of CHO cells constitutively expressing antibody GC-IgG1 and its concentration was determined by the same method as described above.

(22-3) Assessment of isolated antibodies for Ca-dependent human GPC3-binding activity

Isolated antibodies were subjected to ELISA using the following procedure. StreptaWell 96-well microtiter plate (Roche) was coated overnight with 100 µl of PBS containing a biotin-labeled antigen. After the antigen was washed off from each well of the plate using ACES buffer (10 mM ACES, 150 mM NaCl, 100 mM $CaCl_2$, 0.05% Tween20, pH 7.4), the wells were blocked for one hour or more with 250 µl of an ACES Buffer containing 2% BSA. After removing the ACES Buffer containing 2% BSA from each well, a purified IgG serially diluted at a dilution ratio of 4 starting from 10 µg/ml was prepared in advance and aliquoted at 100 µl into the plate. The plate was allowed to stand for one hour to allow binding of IgG to the antigen in each well. Following wash with the ACES Buffer, "10 mM ACES, 150 mM NaCl, 1.2

mM $CaCl_2$, pH 7.4", "10 mM ACES, 150 mM NaCl, 3μM $CaCl_2$, pH 7.4", "10 mM ACES, 150 mM NaCl, 1.2 mM $CaCl_2$, pH 5.8", or "10 mM ACES, 150 mM NaCl, 3 μM $CaCl_2$, pH 5.8" was added to each well. The plate was incubated at 37°C for 30 minutes. After washing with the ACES Buffer, an HRP-conjugated anti-human IgG antibody (BIOSOURCE) diluted with an ACES Buffer containing 2% BSA was added to each well. The plate was incubated for one hour. Following wash with ACES Buffer, the TMB single solution (ZYMED) was added to each well. The chromogenic reaction in the solution of each well was terminated by adding sulfuric acid. Then, the developed color was assessed by measuring absorbance at 450 nm.

The measurement result is shown in Fig. 29. In the case of GC-IgG1, the absorbance of GC-IgG1 did not change according to the calcium ion concentration. By contrast, as for CSCM-01_005, CSCM-01_009, CSCM-01_015, and CSCM-01_023, the absorbance was considerably lower at a calcium ion concentration of 3 μM (low calcium ion concentration) than at 1.2 mM (high calcium ion concentration). The result described above demonstrates that CSCM-01_005, CSCM-01_009, CSCM-01_015, and CSCM-01_023 have the property that their antigen binding varies according to the calcium ion concentration. This demonstrates that calcium-dependent antibodies against human glypican 3 are also obtainable. As compared to typical anti-human glypican 3 antibodies, it is considered that the calcium-dependent anti-human glypican 3 antibodies can accelerate elimination of human glypican 3, similarly to the case with human IL-6R, human CD4, or human IgA described in Examples above. Moreover, it is considered that the elimination of human glypican 3 can be further accelerated by enhancing the FcRn binding of the calcium-dependent anti-human glypican 3 antibodies at pH 7.4.

[Example 23] Antibodies that bind to IgE in a calcium-dependent manner

(23-1) Preparation of biotinylated human IgE

Human IgE was prepared as an antigen by the following procedure. An animal cell expression vector inserted with a DNA sequence encoding IgE-H (SEQ ID NO: 104, a sequence for biotinylation is linked at the C terminus) and L(WT) (SEQ ID NO: 14) was prepared. Using the expression vector and FreeStyle293 (Invitrogen), the full-length human IgE protein to which a sequence for biotinylation is linked to the C terminus was expressed in the culture supernatant. From the isolated culture supernatant, a biotinylated human IgE was prepared by performing ion-exchange chromatography, avidin-affinity purification, and gel filtration chromatography purification.

(23-2) Expression and purification of antibodies that bind to human IgE

GEB0100 (heavy chain, SEQ ID NO: 105; light chain, SEQ ID NO: 106), GEB0220 (heavy chain, SEQ ID NO: 107; light chain, SEQ ID NO: 108), GEB0230 (heavy chain, SEQ ID

NO: 109; light chain, SEQ ID NO: 110), and Xolair (heavy chain, SEQ ID NO: 111; light chain, SEQ ID NO: 112) were antibodies that bind to human IgE. GEB0100 (heavy chain, SEQ ID NO: 105; light chain, SEQ ID NO: 106), GEB0220 (heavy chain, SEQ ID NO: 107; light chain, SEQ ID NO: 108), GEB0230 (heavy chain, SEQ ID NO: 109; light chain, SEQ ID NO: 110), and Xolair (generic name: Omalizumab) (heavy chain, SEQ ID NO: 111; light chain, SEQ ID NO: 112) were each inserted into animal expression plasmids by a method known to those skilled in the art. Antibodies were expressed by the following procedure. The constructed plasmids were introduced into cells of human fetal kidney cell-derived FreeStyle 293-F (Invitrogen) by a lipofection method. The cells were cultured for four to seven days in a CO_2 incubator (37°C, 8% CO_2, 90 rpm). From the prepared culture supernatants, antibodies were purified using the rProtein A Sepharose™ Fast Flow (Amersham Biosciences) by a method known to those skilled in the art.

The concentrations of purified antibodies were determined by measuring absorbance at 280 nm using a spectrophotometer. Antibody concentrations were calculated from the determined values using an extinction coefficient calculated by the PACE method (Protein Science (1995) 4: 2411-2423).

(23-3) Assessment of isolated antibodies for Ca-dependent human IgE-binding activity

Isolated antibodies were assessed for their Ca-dependent binding activity to human IgE by ELISA. Specifically, 40 µl of 1 µg/ml Goat anti-rabbit IgG-Fc polyclonal antibody (Bethyl laboratory; A120-111A) or 1 µg/ml Goat anti-human IgG-Fc polyclonal antibody (ICN biomedicals; 55071) was added to the NUNC Immuno 384-well Plate MaxiSorp (Thermo fisher scientific; 464718). After one hour of incubation at room temperature, the solution was removed and 50 µl of Blocking One Reagent (Nacalai Tesque; 03953-95) diluted to 20% was added. After one hour of incubation at room temperature, the solution was removed and 40 µl of purified antibodies diluted with Tris buffer containing 1.2 mM calcium chloride were added. After overnight incubation at 4°C, the plate was washed three times with 80 µl of Tris buffer containing 1.2 mM calcium chloride and 0.05%(w/v) Tween-20, and 40 µl of the biotinylated human IgE (prepared as described in (23-1)) diluted to 500 ng/ml with a Tris buffer containing 1.2 mM calcium chloride was added. After one hour of incubation at room temperature, the plate was washed three times with 80 µl of a Tris buffer containing 1.2 mM calcium chloride and 0.05%(w/v) Tween-20. 80 µl of an ACES buffer (pH 7.4) containing 2 mM or 3 µM calcium chloride was added and then immediately removed. Again, 80 µl of an ACES buffer (pH 7.4) containing 2 mM or 3 µM calcium chloride was added to the plate. After one hour of incubation at 37°C, the plate was washed three times with 80 µl of a Tris buffer containing 1.2 mM calcium chloride and 0.05%(w/v) Tween-20, and 40 µl of HRP-labeled streptavidin (Thermo fisher

scientific; 21132) diluted to 25 ng/ml with a Tris buffer containing 1.2 mM calcium chloride was added. After one hour of incubation at room temperature, the plate was washed three times with 80 µl of a Tris buffer containing 1.2 mM calcium chloride and 0.05%(w/v) Tween-20. Then, 40 µl of a chromogenic substrate (KPL; 50-66-06: ABTS peroxidase substrate system 1 component) is added. Following 15 to 30 minutes of incubation at room temperature, the absorbance at 405 nm was measured (Molecular devices; SpectraMax Plus384).

The measurement result is shown in Fig. 30. In the case of Xolair, the absorbance did not change according to the calcium ion concentration. By contrast, as for GEB0100, GEB0220, and GEB0230, the absorbance was considerably lower at a calcium ion concentration of 3 µM (low calcium ion concentration) than at 1.2 mM (high calcium ion concentration). The result described above demonstrates that GEB0100, GEB0220, and GEB0230 have the property that their antigen binding varies according to the calcium ion concentration. This indicates that calcium-dependent antibodies against human IgE are also obtainable. As compared to typical anti-human IgE antibodies such as Xolair, it is considered that the calcium-dependent anti-human IgE antibodies can accelerate the elimination of human IgE, similarly to the case with human IL-6R, human CD4, or human IgA described in Examples above. Moreover, it is considered that the elimination of human IgE can be further accelerated by enhancing the FcRn binding of the calcium-dependent anti-human IgE antibodies at pH 7.4.

[Reference Example 1] Preparation of soluble human IL-6 receptor (hsIL-6R)

Recombinant human IL-6 receptor as an antigen was prepared as follows. A CHO cell line constitutively expressing soluble human IL-6 receptor (hereinafter referred to as hsIL-6R) having the amino acid sequence of positions 1 to 357 from the N terminus as reported in J. Immunol. 152: 4958-4968 (1994) was established by a method known to those skilled in the art. The cells were cultured to express hsIL-6R. The hsIL-6R was purified from the culture supernatant by two steps: Blue Sepharose 6 FF column chromatography and gel filtration column chromatography. A fraction eluted as the main peak in the final stage was prepared as the final purification product.

[Reference Example 2] Preparation of human FcRn

FcRn is a complex of FcRn and β2-microglobulin. Oligo-DNA primers were prepared based on the published human FcRn gene sequence (J Exp Med. 1994 Dec 1; 180(6): 2377-81). A DNA fragment encoding the whole gene was prepared by PCR using human cDNA (Human Placenta Marathon-Ready cDNA, Clontech) as a template and the prepared primers. Using the obtained DNA fragment as a template, a DNA fragment encoding the extracellular domain containing the signal region (Met1-Leu290) was amplified by PCR, and inserted into a

mammalian cell expression vector. Likewise, oligo-DNA primers were prepared based on the published human β2-microglobulin gene sequence (Proc. Natl. Acad. Sci. U.S.A. 99 (26): 16899-16903 (2002)). A DNA fragment encoding the whole gene was prepared by PCR using human cDNA (Human Placenta Marathon-Ready cDNA, Clontech) as a template and the prepared primers. Using the obtained DNA fragment as a template, a DNA fragment encoding the whole protein containing a signal region (Met1-Met119) was amplified by PCR and inserted into a mammalian cell expression vector.

Soluble human FcRn was expressed by the following procedure. The plasmids constructed for expressing human FcRn (SEQ ID NO: 17) and β2-microglobulin (SEQ ID NO: 18) were introduced into cells of the human embryonic kidney cancer-derived cell line HEK293H (Invitrogen) by the lipofection method using PEI (Polyscience). The resulting culture supernatant was collected, and FcRn was purified using IgG Sepharose 6 Fast Flow (Amersham Biosciences), followed by further purification using HiTrap Q HP (GE Healthcare) (J Immunol. 2002 Nov 1; 169(9): 5171-80).

[Reference Example 3] Studies to improve the antigen elimination-accelerating effect of pH-dependent antigen-binding antibodies (*in vivo* test)

(3-1) Preparation of pH-dependent human IL-6 receptor-binding antibodies that bind to FcRn under neutral condition

Mutations were introduced into Fv4-IgG1 comprising VH3-IgG1 (SEQ ID NO: 19) and VL3-CK (SEQ ID NO: 20) to augment the FcRn binding under a neutral condition (pH 7.4). Specifically, VH3-IgG1-v1 (SEQ ID NO: 21) was prepared from the heavy chain constant region of IgG1 by substituting Tyr for Met at position 252, Thr for Ser at position 254, and Glu for Thr at position 256 in EU numbering, while VH3-IgG1-v2 (SEQ ID NO: 22) was constructed from the heavy chain constant region of IgG1 by substituting Trp for Asn at position 434 in EU numbering. The mutants were constructed by amino acid substitution using QuikChange Site-Directed Mutagenesis Kit (Stratagene) or In-Fusion HD Cloning Kit (Clontech) according to the method described in the provided manual. The prepared plasmid fragments were inserted into animal cell expression vectors to construct expression vectors for the H chain and L chain of interest. The nucleotide sequences of the constructed expression vectors were determined by a method known to those skilled in the art.

H54/L28-IgG1 comprising H54 (SEQ ID NO: 5) and L28 (SEQ ID NO: 6), Fv4-IgG1 comprising VH3-IgG1 (SEQ ID NO: 19) and VL3-CK (SEQ ID NO: 20), Fv4-IgG1-v1 comprising VH3-IgG1-v1 (SEQ ID NO: 21) and VL3-CK (SEQ ID NO: 20), and Fv4-IgG1-v2 comprising VH3-IgG1-v2 (SEQ ID NO: 22) and VL3-CK (SEQ ID NO: 20) were expressed and purified by the method described below. Antibodies were expressed by FreestyleHEK293

(Invitrogen) as described by the protocol provided by the manufacture or HEK293H cell line (Invitrogen). Human embryonic kidney cancer-derived HEK293H cell line (Invitrogen) was suspended in DMEM (Invitrogen) supplemented with 10% Fetal Bovine Serum (Invitrogen). The cells were plated at 10 ml per dish in dishes for adherent cells (10 cm in diameter; CORNING) at a cell density of 5 to 6 x 10^5 cells/ml and cultured in a CO_2 incubator (37°C, 5% CO_2) for one whole day and night. Then, the medium was removed by aspiration, and 6.9 ml of CHO-S-SFM-II medium (Invitrogen) was added. The prepared plasmid was introduced into the cells by the lipofection method. The resulting culture supernatants were collected, centrifuged (approximately 2,000 x g, 5 min, room temperature) to remove cells, and sterilized by filtering through 0.22-μm filter MILLEX (registered trademark)-GV (Millipore) to obtain the supernatants. Antibodies were purified from the obtained culture supernatants by a method known to those skilled in the art using rProtein A Sepharose™ Fast Flow (Amersham Biosciences). To determine the concentration of the purified antibody, absorbance was measured at 280 nm using a spectrophotometer. Antibody concentrations were calculated from the determined values using an absorbance coefficient calculated by the method described in Protein Science (1995) 4: 2411-2423.

(3-2) *In vivo* test using human FcRn transgenic mice and normal mice

The *in vivo* kinetics of hsIL-6R (soluble human IL-6 receptor: prepared as described in Reference Example 1) and anti-human IL-6 receptor antibody was assessed after administering hsIL-6R alone or hsIL-6R and anti-human IL-6 receptor antibody in combination to human FcRn transgenic mice (B6.mFcRn-/-.hFcRn Tg line 276 +/+ mouse, Jackson Laboratories; Methods Mol Biol. (2010) 602: 93-104) and normal mice (C57BL/6J mouse; Charles River Japan). An hsIL-6R solution (5 μg/ml) or a solution of mixture containing hsIL-6R and anti-human IL-6 receptor antibody (5 μg/ml and 0.1 mg/ml, respectively) was administered once at a dose of 10 ml/kg into the caudal vein. In this case, the anti-human IL-6 receptor antibody is present in excess over hsIL-6R, and therefore almost every hsIL-6R is assumed to be bound to the antibody. Blood was collected 15 minutes, seven hours, one day, two days, three days, four days, seven days, 14 days, 21 days, and 28 days after administration. The collected blood was immediately centrifuged at 15,000 rpm and 4°C for 15 minutes to separate the plasma. The separated plasma was stored in a refrigerator at or below -20°C before assay. The anti-human IL-6 receptor antibodies used are: above-described H54/L28-IgG1, Fv4-IgG1, and Fv4-IgG1-v2 for human FcRn transgenic mice, and above-described H54/L28-IgG1, Fv4-IgG1, Fv4-IgG1-v1, and Fv4-IgG1-v2 for normal mice.

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

The concentration of anti-human IL-6 receptor antibody in mouse plasma was measured by ELISA. Anti-human IgG (γ chain specific) F(ab')2 antibody fragment (Sigma) was dispensed onto a Nunc-ImmunoPlate MaxiSorp (Nalge Nunc International) and allowed to stand overnight at 4°C to prepare anti-human IgG-immobilized plates. Calibration curve samples having plasma concentrations of 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, and 0.0125 μg/ml, and mouse plasma samples diluted 100-fold or more were prepared. 200 μL of 20 ng/ml hsIL-6R was added to 100 μL of the calibration curve samples and plasma samples, and then the samples were allowed to stand for one hour at room temperature. Subsequently, the samples were dispensed into the anti-human IgG-immobilized plates, and allowed to stand for one hour at room temperature. Then, Biotinylated Anti-Human IL-6R Antibody (R&D) was added to react for one hour at room temperature. Subsequently, Streptavidin-PolyHRP80 (Stereospecific Detection Technologies) was added to react for one hour at room temperature, and chromogenic reaction was carried out using TMP One Component HRP Microwell Substrate (BioFX Laboratories) as a substrate. After stopping the reaction with 1N sulfuric acid (Showa Chemical), the absorbance at 450 nm was measured by a microplate reader. The concentration in mouse plasma was calculated from the absorbance of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices). The time course of plasma concentration after intravenous administration as measured by this method is shown in Fig. 31 for human FcRn transgenic mice and Fig.33 for normal mice.

(3-4) Measurement of hsIL-6R plasma concentration by electrochemiluminescence assay

The concentration of hsIL-6R in mouse plasma was measured by electrochemiluminescence. hsIL-6R calibration curve samples adjusted to concentrations of 2,000, 1,000, 500, 250, 125, 62.5, and 31.25 pg/ml, and mouse plasma samples diluted 50-fold or more were prepared. The samples were mixed with a solution of Monoclonal Anti-human IL-6R Antibody (R&D) ruthenium-labeled with Sulfo-Tag NHS Ester (Meso Scale Discovery), Biotinylated Anti-human IL-6R Antibody (R&D), and WT-IgG1, and then allowed to react overnight at 37°C. The final concentration of WT-IgG1 as an anti-human IL-6 receptor antibody, comprising H (WT) (SEQ ID NO: 13) and L (WT) (SEQ ID NO: 14), was 333 μg/ml, which is in excess of the concentration of anti-human IL-6 receptor antibody contained in the samples, for the purpose of binding nearly all of the hsIL-6R molecules in the samples to WT-IgG1. Subsequently, the samples were dispensed into an MA400 PR Streptavidin Plate (Meso Scale Discovery), and allowed to react for one hour at room temperature, and washing was performed. Immediately after Read Buffer T (x4) (Meso Scale Discovery) was dispensed, the measurement was performed by the Sector PR 400 Reader (Meso Scale Discovery). The hsIL-6R concentration was calculated based on the response of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices). The time course of plasma hsIL-6R

concentration after intravenous administration as measured by this method is shown in Fig. 32 for human FcRn transgenic mice and Fig. 34 for normal mice.

(3-5) Determination of free hsIL-6R concentration in plasma by electrochemiluminescence assay

To assess the degree of neutralization of soluble human IL-6 receptor in plasma, the concentration of soluble human IL-6 receptor free of (non-neutralized by) anti-human IL-6 receptor antibody (free hsIL-6R concentration) in mouse plasma was determined by electrochemiluminescence assay. All IgG-type antibodies (mouse IgG, anti-human IL-6 receptor antibody, and anti-human IL-6 receptor antibody-soluble human IL-6 receptor complex) in plasma were adsorbed onto protein A by adding 12 µl each of hsIL-6R standard samples prepared at 10,000, 5,000, 2,500, 1,250, 625, 312.5, or 156.25 pg/ml and mouse plasma samples onto an appropriate amount of rProtein A Sepharose Fast Flow (GE Healthcare) resin dried on 0.22-µm filter cup (Millipore). Then, the solution in a cup was spun down using a high-speed centrifuge to collect the solution that passed through. The passed-through solution does not contain Protein A-bound anti-human IL-6 receptor antibody-soluble human IL-6 receptor complex. Thus, the concentration of free hsIL-6R in plasma can be determined by measuring the concentration of hsIL-6R in the passed-through solution. Then, the passed-through solution was mixed with a monoclonal anti-human IL-6R antibody (R&D) ruthenium-labeled with SULFO-TAG NHS Ester (Meso Scale Discovery) and a biotinylated anti-human IL-6 R antibody (R&D). The resulting mixture was incubated at room temperature for one hour, and then aliquoted to MA400 PR Streptavidin Plate (Meso Scale Discovery). After another hour of incubation at room temperature, the plate was washed and Read Buffer T (x4) (Meso Scale Discovery) was aliquoted thereto. Immediately, the plate was measured in SECTOR PR 400 reader (Meso Scale Discovery). The hsIL-6R concentration was calculated based on the response in the standard curve using the analysis software SOFTmax PRO (Molecular Devices). A time course of free hsIL-6R concentration in the plasma of normal mice after intravenous administration determined by the above-described method is shown in Fig. 35.

(3-6) Effect of pH-dependent binding to human IL-6 receptor

H54/L28-IgG1 and Fv4-IgG1 which binds to human IL-6 receptor in a pH-dependent manner were tested *in vivo*, and the results were compared between them. As shown in Figs. 31 and 33, the antibody retention in plasma was comparable. Meanwhile, as shown in Figs. 32 and 34, hsIL-6R simultaneously administered with Fv4-IgG1 which binds to human IL-6 receptor in a pH-dependent manner was found to accelerate the elimination of hsIL-6R as compared to hsIL-6R simultaneously administered with H54/L28-IgG1. The above tendency was observed in both human FcRn transgenic and normal mice; thus, it was demonstrated that by conferring a pH-

dependent human IL-6 receptor-binding ability, the plasma hsIL-6R concentration four days after administration could be decreased by about 17 and 34 times, respectively.

(3-7) Effect of FcRn binding under neutral condition (pH 7.4)

Natural human IgG1 has been reported to hardly bind to (have extremely low affinity for) human FcRn under a neutral condition (pH 7.4). The human FcRn binding under a neutral condition (pH 7.4) was reported to be augmented by substituting Trp for Asn at position 434 (EU numbering) in natural human IgG1 (J Immunol. (2009) 182 (12): 7663-71). Fv4-IgG1-v2 which results from introducing the above amino acid substitution into Fv4-IgG1 was tested by an *in vivo* test using human FcRn transgenic mice. The test result was compared to that of Fv4-IgG1. As shown in Fig. 31, the antibody plasma retention was comparable between the two. Meanwhile, as shown in Fig. 32, hsIL-6R simultaneously administered with Fv4-IgG1-v2 that exhibits enhanced human FcRn binding under a neutral condition (pH 7.4) was found to be eliminated faster as compared to hsIL-6R simultaneously administered with Fv4-IgG1. Thus, it was demonstrated that by conferring the ability to bind to human FcRn under a neutral condition (pH 7.4), the plasma concentration of hsIL-6R four days after administration could be reduced by about four times.

Based on the homology between human FcRn and mouse FcRn, the substitution of Trp for Asn at position 434 in EU numbering is assumed to augment the binding to mouse FcRn under a neutral condition (pH 7.4). Meanwhile, the binding to mouse FcRn under a neutral condition (pH 7.4) has been reported to be augmented by substituting Tyr for Met at position 252, Thr for Ser at position 254, and Glu for Thr at position 256 in EU numbering (J Immunol. (2002) 169(9): 5171-80). Fv4-IgG1-v1 and Fv4-IgG1-v2 which result from introducing the above-described amino acid substitutions into Fv4-IgG1 were tested in vivo using normal mice. The test results were compared to that of Fv4-IgG1. As shown in Fig. 33, the plasma retention times of Fv4-IgG1-v1 and Fv4-IgG1-v2 which had also been improved to increase the binding to mouse FcRn under a neutral condition (pH 7.4) were slightly shortened (the neutralizing antibody concentrations in plasma one day after administration were reduced by about 1.5 and 1.9 times, respectively) as compared to Fv4-IgG1.

As shown in Fig. 34, hsIL-6R simultaneously administered with Fv4-IgG1-v1 or Fv4-IgG1-v2 which had been improved to increase the binding to mouse FcRn under a neutral condition (pH 7.4) was demonstrated to be eliminated markedly faster as compared to hsIL-6R simultaneously administered with Fv4-IgG1. Fv4-IgG1-v1 and Fv4-IgG1-v2 reduced the plasma hsIL-6R concentrations one day after administration by about 32 and 80 times, respectively. Thus, it was revealed that the plasma concentration could be reduced by conferring mouse FcRn-binding ability under a neutral condition (pH 7.4). As described above, by conferring the mouse

FcRn-binding ability under a neutral condition (pH 7.4), the plasma antibody concentration was slightly reduced; however, the effect of reducing the plasma hsIL-6R concentration, which largely exceeded the decrease in antibody concentration, was produced. Furthermore, hsIL-6R simultaneously administered with Fv4-IgG1-v1 or Fv4-IgG1-v2 was found to be eliminated faster even when compared to the group administered with hsIL-6R alone. As shown in Fig. 34, it was demonstrated that hsIL-6R simultaneously administered with Fv4-IgG1-v1 or Fv4-IgG1-v2 could reduce the plasma hsIL-6R concentration one day after administration by about 4 or 11 times, respectively, as compared to hsIL-6R alone. Specifically, this means that the elimination of soluble IL-6 receptor could be accelerated by administering the antibody that binds to soluble IL-6 receptor in a pH-dependent manner and which is conferred with mouse FcRn-binding ability under a neutral condition (pH 7.4). Specifically, the plasma antigen concentration can be reduced in vivo by administering such an antibody to the body.

As shown in Fig. 35, free hsIL-6R was in a detectable concentration range for seven days after administration of H54/L28-IgG1, while free hsIL-6R was undetectable after one day following administration of Fv4-IgG1. On the other hand, free hsIL-6R was not detectable after seven hours following administration of Fv4-IgG1-v1 or Fv4-IgG1-v2. Specifically, the free hsIL-6R concentration was lower in the presence of Fv4-IgG1 that binds to hsIL-6R in a pH-dependent manner as compared to H54/L28-IgG1, suggesting that a strong hsIL-6R-neutralizing effect was produced by conferring the pH-dependent hsIL-6R-binding ability. Furthermore, the free hsIL-6R concentration was much lower in the presence of Fv4-IgG1-v1 or Fv4-IgG1-v2, both of which were modified from Fv4-IgG1 to increase the FcRn-binding ability at pH 7.4. This demonstrates that a much stronger hsIL-6R-neutralizing effect can be produced by increasing the FcRn-binding ability at pH 7.4.

When administered, an ordinary neutralizing antibody such as H54/L28-IgG1 reduces the clearance of a binding antigen, resulting in prolonged antigen plasma retention. It is not preferred that administered antibodies prolong the plasma retention of an antigen whose action is intended to be neutralized by the antibodies. The antigen plasma retention can be shortened by conferring the pH dependency to antigen binding (the antibody binds under neutral conditions but is dissociated under acidic conditions). In the present invention, the antigen retention time in plasma could be further shortened by additionally conferring human FcRn-binding ability under a neutral condition (pH 7.4). Furthermore, it was demonstrated that as compared to clearance of antigen alone, antigen clearance could be increased by administering an antibody that binds to an antigen in a pH dependent manner, and which is conferred with FcRn-binding ability under a neutral condition (pH 7.4). To date, there is no method available for increasing antigen clearance by antibody administration relative to clearance of antigen alone. Thus, the methods established as described in this EXAMPLE are very useful as a method for eliminating antigens

from plasma by administering antibodies. Furthermore, the present inventors discovered for the first time the advantage of increasing the FcRn-binding ability under a neutral condition (pH 7.4). Furthermore, both v4-IgG1-v1 and Fv4-IgG1-v2 which have different amino acid substitutions that increase the FcRn-binding ability under a neutral condition (pH 7.4) produced comparable effects. This suggests that regardless of the type of amino acid substitution, every amino acid substitution that increases the human FcRn-binding ability under a neutral condition (pH 7.4) potentially has an effect of accelerating antigen elimination. Specifically, antibody molecules that eliminate antigens from plasma when administered can be produced using the following amino acid substitutions alone or in combination:

an amino acid substitution of Ile for Pro at position 257 and an amino acid substitution of Ile for Gln at position 311 in EU numbering, both of which have been reported in J Biol Chem. 2007, 282(3): 1709-17; an amino acid substitution of Ala, Tyr, or Trp for Asn at position 434, an amino acid substitution of Tyr for Met at position 252, an amino acid substitution of Gln for Thr at position 307, an amino acid substitution of Pro for Val at position 308, an amino acid substitution of Gln for Thr at position 250, an amino acid substitution of Leu for Met at position 428, an amino acid substitution of Ala for Glu at position 380, an amino acid substitution of Val for Ala at position 378, an amino acid substitution of Ile for Tyr at position 436 in EU numbering, all of which have been reported in J Immunol. (2009) 182(12): 7663-71; an amino acid substitution of Tyr for Met at position 252, an amino acid substitution of Thr for Ser at position 254, an amino acid substitution of Glu for Thr at position 256 in EU numbering, all of which have been reported in J Biol Chem. 2006 Aug. 18, 281(33): 23514-24; an amino acid substitution of Lys for His at position 433, an amino acid substitution of Phe for Asn at position 434, and an amino acid substitution of His for Tyr at position 436 in EU numbering, all of which have been reported in Nat Biotechnol. 2005 Oct. 23(10): 1283-8; and the like.

[Reference Example 4] Assessment of human FcRn-binding activity

For the Biacore-based assay system for testing the interaction between antibody and FcRn, a system that immobilizes antibody on a sensor chip and uses human FcRn as an analyte is reported in J Immunol. (2009) 182(12): 7663-71. For this purpose, human FcRn was prepared as described in Reference Example 2. Fv4-IgG1, Fv4-IgG1-v1, and Fv4-IgG1-v2 were assessed for the human FcRn-binding activity (dissociation constant (KD)) at pH 6.0 and pH 7.4 by using the above-described system. The antibodies were tested as a test substance after direct immobilization onto Series S Sensor Chip CM5. Using an amino-coupling kit according to the supplier's instruction manual, the antibodies were immobilized onto Sensor Chip so as to secure an immobilization amount of 500 RU. The running buffer used was 50 mmol/l Na-phosphate/150 mmol/l NaCl containing 0.05% (v/v%) Surfactant P20 (pH 6.0).

With the prepared sensor chips, assay was carried out using as a running buffer, 50 mmol/l Na-phosphate/150 mmol/l NaCl containing 0.05% Surfactant P20 (pH 6.0) or 50 mmol/l Na-phosphate/150 mmol/l NaCl containing 0.05% Surfactant P20 (pH 7.4). Assays were carried out exclusively at 25°C. The diluted human FcRn solutions and running buffer as a reference solution were injected at a flow rate of 5 μl/min for ten minutes to allow for human FcRn to interact with the antibody on the chip. Next, the running buffer was injected at a flow rate of 5 μl /min for one minute to monitor the dissociation of FcRn. Then, the sensor chip was regenerated by two rounds of injection of 20 mmol/l Tris-HCl/150 mmol/l NaCl (pH 8.1) at a flow rate of 30 μl /min for 15 seconds.

The assay results were analyzed using Biacore T100 Evaluation Software (Ver. 2.0.1). By a steady-state affinity method, the dissociation constant (KD) was calculated from the assay results at six different FcRn concentrations. The results on the human FcRn-binding activities (dissociation constants (KD)) of Fv4-IgG1, Fv4-IgG1-v1, and Fv4-IgG1-v2 at pH 6.0 and pH 7.4 are shown in Table 24 below.

[Table 24]

	KD (μM)	
	pH6.0	pH7.4
Fv4-IgG1	1.99	NA
Fv4-IgG1-v1	0.32	36.55
Fv4-IgG1-v2	0.11	11.03

At pH 7.4, the binding of human FcRn to Fv4-IgG1 was too weak to determine the KD value (NA). Meanwhile, Fv4-IgG1-v1 and Fv4-IgG1-v2 were observed to bind to human FcRn at pH 7.4, and the KD values were determined to be 36.55 and 11.03 μM, respectively. The KD values for human FcRn at pH 6.0 were determined to be 1.99, 0.32, and 0.11 μM. As shown in Fig. 31, when compared to Fv4-IgG1, Fv4-IgG1-v2 accelerated the elimination of hsIL-6R in human FcRn transgenic mice. Thus, antigen elimination can be predicted to be accelerated by augmenting the human FcRn binding at pH 7.4 at least to be stronger than 11.03 μM by alteration of human IgG1. Meanwhile, as described in J Immunol. (2002) 169(9): 5171-80, human IgG1 binds about ten times more strongly to mouse FcRn than human FcRn. For this reason, Fv4-IgG1-v1 and Fv4-IgG1-v2 are also predicted to bind about ten times more strongly

to mouse FcRn than human FcR at pH 7.4. Acceleration of the hsIL-6R elimination by Fv4-IgG1-v1 or Fv4-IgG1-v2 in normal mice shown in Fig. 34 is more significant than acceleration of the elimination by Fv4-IgG1-v2 in human FcRn transgenic mice shown in Fig. 32. This suggests that the degree of acceleration of hsIL-6R elimination is increased according to the strength of FcRn binding at pH 7.4.

[Reference Example 5] Preparation of pH-dependent human IL-6 receptor-binding antibodies with enhanced human FcRn binding under neutral condition

(5-1) Preparation of heavy chain constant region mutants of Fv4-IgG1

Various alterations to augment the human FcRn binding under a neutral condition were introduced into Fv4-IgG1 to further enhance the antigen elimination effect of the pH-dependent human IL-6 receptor-binding antibody in human FcRn transgenic mice. Specifically, the amino acid alterations shown in Tables 25-1 and 25-2 were introduced into the heavy chain constant region of Fv4-IgG1 to produce various mutants (amino acid numbers of the mutation sites are presented according to EU numbering). The amino acid substitutions were introduced by methods known to those skilled in the art as described in Reference Example 3.

[Table 25-1]

VARIANT NAME	KD (M)	AMINO ACID ALTERATION
IgG1	ND	NONE
IgG1-v1	3.2E-06	M252Y/S254T/T256E
IgG1-v2	8.1E-07	N434W
IgG1-F3	2.5E-06	N434Y
IgG1-F4	5.8E-06	N434S
IgG1-F5	6.8E-06	N434A
IgG1-F7	5.6E-06	M252Y
IgG1-F8	4.2E-06	M252W
IgG1-F9	1.4E-07	M252Y/S254T/T256E/N434Y
IgG1-F10	6.9E-08	M252Y/S254T/T256E/N434W
IgG1-F11	3.1E-07	M252Y/N434Y
IgG1-F12	1.7E-07	M252Y/N434W
IgG1-F13	3.2E-07	M252W/N434Y
IgG1-F14	1.8E-07	M252W/N434W
IgG1-F19	4.6E-07	P257L/N434Y
IgG1-F20	4.6E-07	V308F/N434Y
IgG1-F21	3.0E-08	M252Y/V308P/N434Y
IgG1-F22	2.0E-06	M428L/N434S
IgG1-F25	9.2E-09	M252Y/S254T/T256E/V308P/N434W
IgG1-F26	1.0E-06	I332V
IgG1-F27	7.4E-06	G237M
IgG1-F29	1.4E-06	I332V/N434Y
IgG1-F31	2.8E-06	G237M/V308F
IgG1-F32	8.0E-07	S254T/N434W
IgG1-F33	2.3E-06	S254T/N434Y
IgG1-F34	2.8E-07	T256E/N434W
IgG1-F35	8.4E-07	T256E/N434Y
IgG1-F36	3.6E-07	S254T/T256E/N434W
IgG1-F37	1.1E-06	S254T/T256E/N434Y
IgG1-F38	1.0E-07	M252Y/S254T/N434W
IgG1-F39	3.0E-07	M252Y/S254T/N434Y
IgG1-F40	8.2E-08	M252Y/T256E/N434W
IgG1-F41	1.5E-07	M252Y/T256E/N434Y
IgG1-F42	1.0E-06	M252Y/S254T/T256E/N434A
IgG1-F43	1.7E-06	M252Y/N434A
IgG1-F44	1.1E-06	M252W/N434A
IgG1-F47	2.4E-07	M252Y/T256Q/N434W
IgG1-F48	3.2E-07	M252Y/T256Q/N434Y
IgG1-F49	5.1E-07	M252F/T256D/N434W
IgG1-F50	1.2E-06	M252F/T256D/N434Y
IgG1-F51	8.1E-06	N434F/Y436H
IgG1-F52	3.1E-06	H433K/N434F/Y436H
IgG1-F53	1.0E-06	I332V/N434W
IgG1-F54	8.4E-08	V308P/N434W
IgG1-F56	9.4E-07	I332V/M428L/N434Y
IgG1-F57	1.1E-05	G385D/Q386P/N389S
IgG1-F58	7.7E-07	G385D/Q386P/N389S/N434W
IgG1-F59	2.4E-06	G385D/Q386P/N389S/N434Y
IgG1-F60	1.1E-05	G385H
IgG1-F61	9.7E-07	G385H/N434W
IgG1-F62	1.9E-06	G385H/N434Y
IgG1-F63	2.5E-06	N434F
IgG1-F64	5.3E-06	N434H

Table 25-2 is the continuation of Table 25-1.

[Table 25-2]

IgG1-F65	2.9E-07	M252Y/S254T/T256E/N434F
IgG1-F66	4.3E-07	M252Y/S254T/T256E/N434H
IgG1-F67	6.3E-07	M252Y/N434F
IgG1-F68	9.3E-07	M252Y/N434H
IgG1-F69	5.1E-07	M428L/N434W
IgG1-F70	1.5E-06	M428L/N434Y
IgG1-F71	8.3E-08	M252Y/S254T/T256E/M428L/N434W
IgG1-F72	2.0E-07	M252Y/S254T/T256E/M428L/N434Y
IgG1-F73	1.7E-07	M252Y/M428L/N434W
IgG1-F74	4.6E-07	M252Y/M428L/N434Y
IgG1-F75	1.4E-06	M252Y/M428L/N434A
IgG1-F76	1.0E-06	M252Y/S254T/T256E/M428L/N434A
IgG1-F77	9.9E-07	T256E/M428L/N434Y
IgG1-F78	7.8E-07	S254T/M428L/N434W
IgG1-F79	5.9E-06	S254T/T256E/N434A
IgG1-F80	2.7E-06	M252Y/T256Q/N434A
IgG1-F81	1.6E-06	M252Y/T256E/N434A
IgG1-F82	1.1E-06	T256Q/N434W
IgG1-F83	2.6E-06	T256Q/N434Y
IgG1-F84	2.8E-07	M252W/T256Q/N434W
IgG1-F85	5.5E-07	M252W/T256Q/N434Y
IgG1-F86	1.5E-06	S254T/T256Q/N434W
IgG1-F87	4.3E-06	S254T/T256Q/N434Y
IgG1-F88	1.9E-07	M252Y/S254T/T256Q/N434W
IgG1-F89	3.6E-07	M252Y/S254T/T256Q/N434Y
IgG1-F90	1.9E-08	M252Y/T256E/V308P/N434W
IgG1-F91	4.8E-08	M252Y/V308P/M428L/N434Y
IgG1-F92	1.1E-08	M252Y/S254T/T256E/V308P/M428L/N434W
IgG1-F93	7.4E-07	M252W/M428L/N434W
IgG1-F94	3.7E-07	P257L/M428L/N434Y
IgG1-F95	2.6E-07	M252Y/S254T/T256E/M428L/N434F
IgG1-F99	6.2E-07	M252Y/T256E/N434H

The variants each comprising a prepared heavy chain and L (WT) (SEQ ID NO: 14) were expressed and purified by methods known to those skilled in the art as described in Reference Example 3.

(5-2) Assessment of human FcRn binding

The binding between antibody and human FcRn was kinetically analyzed using Biacore T100 (GE Healthcare). For this purpose, human FcRn was prepared as described in Reference Example 2. An appropriate amount of protein L (ACTIGEN) was immobilized onto Sensor chip CM4 (GE Healthcare) by the amino coupling method, and the chip was allowed to capture an antibody of interest. Then, diluted FcRn solutions and running buffer (as a reference solution)

were injected to allow human FcRn to interact with the antibody captured on the sensor chip. The running buffer used comprised 50 mmol/l sodium phosphate, 150 mmol/l NaCl, and 0.05% (w/v) Tween20 (pH 7.0). FcRn was diluted using each buffer. The chip was regenerated using 10 mmol/l glycine-HCl (pH 1.5). Assays were carried out exclusively at 25°C. The association rate constant ka (1/Ms) and dissociation rate constant kd (1/s), both of which are kinetic parameters, were calculated based on the sensorgrams obtained in the assays, and KD (M) of each antibody for human FcRn was determined from these values. Each parameter was calculated using Biacore T100 Evaluation Software (GE Healthcare).

The assessment result on the human FcRn binding under a neutral condition (pH 7.0) by Biacore is shown in Tables 6-1 and 6-2. The KD of the natural IgG1 could not be calculated because it exhibited only very weak binding. Thus, the KD is indicated as ND in Table 6-1.

[Reference Example 6] *In vivo* test of pH-dependent human IL-6 receptor-binding antibodies with enhanced human FcRn binding under the neutral condition

pH-dependent human IL-6 receptor-binding antibodies having human FcRn binding ability under a neutral condition were produced using the heavy chains prepared as described in Reference Example 4 to have human FcRn binding ability under a neutral condition. The antibodies were assessed for their *in vivo* antigen elimination effect. Specifically, the antibodies listed below were expressed and purified by methods known to those skilled in the art as described in Reference Example 3:

Fv4-IgG1 comprising VH3-IgG1 and VL3-CK;
Fv4-IgG1-v2 comprising VH3-IgG1-v2 and VL3-CK;
Fv4-IgG1-F14 comprising VH3-IgG1-F14 and VL3-CK;
Fv4-IgG1-F20 comprising VH3-IgG1-F20 and VL3-CK;
Fv4-IgG1-F21 comprising VH3-IgG1-F21 and VL3-CK;
Fv4-IgG1-F25 comprising VH3-IgG1-F25 and VL3-CK;
Fv4-IgG1-F29 comprising VH3-IgG1-F29 and VL3-CK;
Fv4-IgG1-F35 comprising VH3-IgG1-F35 and VL3-CK;
Fv4-IgG1-F48 comprising VH3-IgG1-F48 and VL3-CK;
Fv4-IgG1-F93 comprising VH3-IgG1-F93 and VL3-CK; and
Fv4-IgG1-F94 comprising VH3-IgG1-F94 and VL3-CK.

By the same methods described in Reference Example 3, the prepared pH-dependent human IL-6 receptor-binding antibodies were tested *in vivo* using human FcRn transgenic mice (B6.mFcRn-/-.hFcRn Tg line 276 +/+ mouse, Jackson Laboratories; Methods Mol Biol. (2010) 602: 93-104).

A time course of plasma concentration of soluble human IL-6 receptor after intravenous administration to human FcRn transgenic mice is shown in Fig. 36. The test result showed that the plasma concentration of soluble human IL-6 receptor remained low over time in the presence of any of the pH-dependent human IL-6 receptor-binding antibodies with augmented human FcRn binding under neutral condition, as compared to in the presence of Fv4-IgG1 which has almost no human FcRn binding ability under neutral condition. Among others, antibodies that produced the remarkable effect include, for example, Fv4-IgG1-F14. The plasma concentration of soluble human IL-6 receptor simultaneously administered with Fv4-IgG1-F14 was demonstrated to be reduced by about 54 times one day after administration as compared to that of soluble human IL-6 receptor simultaneously administered with Fv4-IgG1. Furthermore, the plasma concentration of soluble human IL-6 receptor simultaneously administered with Fv4-IgG1-F21 was demonstrated to be reduced by about 24 times seven hours after administration as compared to that of soluble human IL-6 receptor simultaneously administered with Fv4-IgG1. In addition, the plasma concentration of soluble human IL-6 receptor simultaneously administered with Fv4-IgG1-F25 seven hours after administration was below the detection limit (1.56 ng/ml). Thus, Fv4-IgG1-F25 was expected to enable a remarkable reduction of 200 or more times in the concentration of soluble human IL-6 receptor relative to the concentration of soluble human IL-6 receptor simultaneously administered with Fv4-IgG1. The findings described above demonstrate that augmentation of the human FcRn binding of pH-dependent antigen-binding antibodies under a neutral condition is highly effective for enhancing the antigen elimination effect. Meanwhile, the type of amino acid alteration to augment human FcRn binding under neutral condition, which is introduced to enhance the antigen elimination effect, is not particularly limited; and such alterations include those shown in Tables 6-1 and 6-2. The antigen elimination effect can be predicted to be enhanced in vivo by any introduced alteration.

Furthermore, the plasma concentration of soluble human IL-6 receptor simultaneously administered with one of the four types of pH-dependent human IL-6 receptor-binding antibodies, Fv4-IgG1-F14, Fv4-IgG1-F21, Fv4-IgG1-F25, and Fv4-IgG1-F48, remained lower over time than that of soluble human IL-6 receptor administered alone. Such a pH-dependent human IL-6 receptor-binding antibody can be administered to the body where the plasma concentration of soluble human IL-6 receptor is kept constant (steady state) to keep the plasma concentration of soluble human IL-6 receptor lower than the steady-state concentration in plasma. Specifically, the in vivo antigen concentration in plasma can be reduced by administering such an antibody to the body.

[Reference Example 7] Assessment for the effectiveness of low-dose (0.01 mg/kg) Fv4-IgG1-F14

Fv4-IgG1-F14 prepared as described in Reference Example 6 was tested at a low dose (0.01 mg/kg) by the same *in vivo* test method as described in Reference Example 6. The result (shown in Figs. 37 and 38) was compared to that described in Reference Example 6, which was obtained by administering Fv4-IgG1 and Fv4-IgG1-F14 at 1 mg/kg.

The result showed that although the plasma antibody concentration in the group administered with Fv4-IgG1-F14 at 0.01 mg/kg was about 100 times lower as compared to the group administered at 1 mg/kg (Fig. 38), the time courses of plasma concentration of soluble human IL-6 receptor were comparable to each other (Fig. 37). In addition, it was demonstrated that the plasma concentration of soluble human IL-6 receptor seven hours after administration in the group administered with Fv4-IgG1-F14 at 0.01 mg/kg was reduced by about three times as compared to that in the group administered with Fv4-IgG1 at 1 mg/kg. Furthermore, in the presence of Fv4-IgG1-F14, the plasma concentration of soluble human IL-6 receptor was lower over time in both groups administered at different doses when compared to the group administered with soluble human IL-6 receptor alone (Fig. 37).

The finding demonstrates that even when administered at a dose one-hundredth of that of Fv4-IgG1, Fv4-IgG1-F14 which results from modification of Fv4-IgG1 to augment human FcRn binding under a neutral condition effectively reduces the plasma concentration of soluble human IL-6 receptor. Specifically, it is predicted that antigens can be efficiently eliminated even at a lower dose when a pH-dependent antigen-binding antibody is modified to augment its FcRn-binding ability under neutral condition.

[Reference Example 8] *In vivo* test based on the steady-state model using normal mice

(8-1) Assessment of the binding to mouse FcRn under neutral condition

VH3/L (WT)-IgG1 comprising VH3-IgG1 (SEQ ID NO: 19) and L (WT) (SEQ ID NO: 14), VH3/L (WT)-IgG1-v2 comprising VH3-IgG1-v2 (SEQ ID NO: 22) and L (WT) (SEQ ID NO: 14), and VH3/L (WT)-IgG1-F20 comprising VH3-IgG1-F20 (SEQ ID NO: 23) and L (WT) (SEQ ID NO: 14), all of which were prepared as described in Reference Example 5, were assessed for mouse FcRn binding under a neutral condition (pH 7.4) by the method described below.

The binding between antibody and mouse FcRn was kinetically analyzed using Biacore T100 (GE Healthcare). An appropriate amount of protein L (ACTIGEN) was immobilized onto Sensor chip CM4 (GE Healthcare) by the amino coupling method, and the chip was allowed to capture an antibody of interest. Then, diluted FcRn solutions and running buffer (as a reference solution) were injected to allow mouse FcRn to interact with the antibody captured on the sensor chip. The running buffer used contains 50 mmol/l sodium phosphate, 150 mmol/l NaCl, and 0.05% (w/v) Tween20 (pH 7.4). FcRn was diluted using each buffer. The chip was regenerated

using 10 mmol/l glycine-HCl (pH 1.5). Assays were carried out exclusively at 25°C. The association rate constant ka (1/Ms) and dissociation rate constant k_d (1/s), both of which are kinetic parameters, were calculated based on the sensorgrams obtained in the assays, and the KD (M) of each antibody for mouse FcRn was determined from these values. Each parameter was calculated using Biacore T100 Evaluation Software (GE Healthcare).

The result is shown in Table 26 (affinity for mouse FcRn at pH 7.4). VH3/L (WT)-IgG1 (IgG1 in Table 26) whose constant region is of the natural IgG1 exhibited only very weak binding to mouse FcRn. Thus, the KD could not be calculated and is indicated as ND in Table 26. The assay result showed that the altered antibodies with enhanced human FcRn binding under neutral condition also exhibited augmented binding to mouse FcRn under the neutral condition.

[Table 26]

	KD (M)
IgG1	ND
IgG1-v2	1.04E-06
IgG1-F20	1.17E-07

(8-2) *In vivo* test using normal mice with a constant plasma concentration of soluble human IL-6 receptor

Using H54/L28-IgG1, Fv4-IgG1, Fv4-IgG1-v2, and Fv4-IgG1-F20 prepared as described in Example 3 and Reference Example 5, an *in vivo* test was conducted by the method described below.

An infusion pump (MINI-OSMOTIC PUMP MODEL 2004; alzet) containing soluble human IL-6 receptor was implanted under the skin on the back of normal mice (C57BL/6J mice; Charles River Japan) to prepare model animals where the plasma concentration of soluble human IL-6 receptor was kept constant. Anti-human IL-6 receptor antibodies were administered to the model animals to assess the *in vivo* kinetics after administration of soluble human IL-6 receptor. Monoclonal anti-mouse CD4 antibody (R&D) was administered at 20 mg/kg once into the caudal vein to suppress the production of neutralizing antibody against soluble human IL-6 receptor. Then, an infusion pump containing 92.8 μg /ml soluble human IL-6 receptor was implanted under the skin on the back of the mice. Three days after implantation of an infusion pump, anti-human IL-6 receptor antibodies were administered at 1 mg/kg once into the caudal

vein. Blood was collected 15 minutes, seven hours, one day, two days, three days, four days, seven days, 14 days, 21 days, and 28 days after administration of the anti-human IL-6 receptor antibody. The collected blood was immediately centrifuged at 15,000 rpm and 4°C for 15 minutes to separate plasma. The separated plasma was stored in a refrigerator at or below -20°C before assay.

(8-3) Determination of plasma concentration of anti-human IL-6 receptor antibodies by ELISA

The method used was the same as described in Reference Example 3.

(8-4) Determination of plasma hsIL-6R concentration by electrochemiluminescence assay

The method used was the same as described in Example 5.

As shown in Fig. 39, the plasma concentration of soluble human IL-6 receptor was elevated to 650 ng/ml (15 times before administration) when H54/L28-IgG1, a neutralizing antibody against soluble human IL-6 receptor, was administered to normal mice (hsIL-6R group) in which the plasma concentration of soluble human IL-6 receptor was kept constantly at about 40 ng/ml. On the other hand, the plasma concentration of soluble human IL-6 receptor was maintained at about 70 ng/ml in the group administered with Fv4-IgG1 which results from conferring H54/L28-IgG1 with a pH-dependent antigen binding ability. This suggests that the increase in the plasma concentration of soluble human IL-6 receptor caused by administration of H54/L28-IgG1, an ordinary neutralizing antibody, can be suppressed to about one tenth by conferring the pH-dependent binding ability.

Furthermore, the plasma concentration of soluble human IL-6 receptor was demonstrated to be maintained at or below one tenth of the steady-state concentration by administering Fv-IgG1-v2 or Fv-IgG1-F20, both of which resulted from introducing an alteration into a pH-dependent human IL-6 receptor-binding antibody to augment the FcRn binding under neutral condition. When Fv-IgG1-v2 was administered, the plasma concentration of soluble human IL-6 receptor 14 days after administration was about 2 ng/ml. Thus, Fv-IgG1-v2 could reduce the concentration to 1/20 of the level before administration. Meanwhile, when Fv-IgG1-F20 was administered, the plasma concentrations of soluble human IL-6 receptor seven hours, one day, two days, and four days after administration were below the detection limit (1.56 ng/ml). This suggests that Fv-IgG1-F20 reduced the concentration to or below 1/25 of the level before administration.

The findings described above demonstrate that the plasma antigen concentration can be significantly reduced by increasing the antigen elimination rate in plasma, by administering an antibody having both pH-dependent antigen-binding ability and FcRn-binding ability under the neutral condition to model animals in which the plasma antigen concentration is kept constant.

Common antibodies such as H54/L28-IgG1 can only neutralize the action of a target antigen by binding to the target antigen, and even worse they increase the plasma antigen concentration. By contrast, antibodies having both pH-dependent antigen-binding ability and FcRn-binding ability under neutral condition were found to be able to not only neutralize the target antigen but also reduce the plasma concentration of the target antigen. The effect of antigen removal from the plasma can be expected to be more beneficial than neutralization. In addition, antigen removal can also work for target antigens that are insufficiently effective by neutralization alone.

[Reference Example 9] Identification of threshold of the binding affinity to human FcRn at neutral pH required to enhance antigen elimination and relationship between antigen elimination and the binding affinity to human FcRn at neutral pH

(9-1) Antibody preparation for *in vivo* study

Fc variants of Fv4-IgG1 comprising VH3-IgG1 (SEQ ID NO: 19) and VL3-CK (SEQ ID NO: 20) with increased FcRn binding under the neutral pH were generated. Specifically, VH3-M73 (SEQ ID NO: 24) and VH3-IgG1-v1 (SEQ ID NO: 21) was prepared. The amino acid substitutions were introduced by methods known to those skilled in the art as described in Reference Example 3.

H54/L28-IgG1 comprising H54 (SEQ ID NO: 5) and L28 (SEQ ID NO: 6), Fv4-IgG1 comprising VH3-IgG1 (SEQ ID NO: 19) and VL3-CK (SEQ ID NO: 20), Fv4-M73 comprising VH3-M73 (SEQ ID NO: 24) and VL3-CK (SEQ ID NO: 20), Fv4-IgG1-v1 comprising VH3-IgG1-v1 (SEQ ID NO: 21) and VL3-CK (SEQ ID NO: 20), and Fv4-IgG1-v2 comprising VH3-IgG1-v2 (SEQ ID NO: 22) and VL3-CK (SEQ ID NO: 20), were expressed and purified by the method known to those skilled in the art described in Reference Example 3.

(9-2) Assessment of the binding affinity of antibodies to human FcRn under neutral pH condition

VH3/L (WT)-IgG1 comprising VH3-IgG1 (SEQ ID NO: 19) and L (WT) (SEQ ID NO: 14), VH3/L (WT)-M73 comprising VH3-M73 (SEQ ID NO: 24) and L (WT) (SEQ ID NO: 14), VH3/L (WT)-IgG1-v1 comprising VH3-IgG1-v1 (SEQ ID NO: 21) and L (WT) (SEQ ID NO: 14), and VH3/L (WT)-IgG1-v2 comprising VH3-IgG1-v2 (SEQ ID NO: 22) and L (WT) (SEQ ID NO: 14), all of which were prepared as described in Reference Example 3, were assessed for human FcRn binding under a neutral pH (pH 7.0).

The binding activity of VH3/L (WT)-IgG1-v1 and VH3/L (WT)-IgG1-v2 to human FcRn was measured using the method described in Reference Example 5. Due to the low binding activity of VH3/L (WT)-IgG1 and VH3/L (WT)-M73 to human FcRn, binding activity to human FcRn could not be measured using the method described in Example 5, therefore, these

antibodies were assessed by the method described below. The binding between antibody and human FcRn was kinetically analyzed using Biacore T100 (GE Healthcare). An appropriate amount of protein L (ACTIGEN) was immobilized onto Sensor chip CM4 (GE Healthcare) by the amine-coupling method, and the chip was allowed to capture an antibody of interest. Then, diluted FcRn solutions and running buffer as a reference solution were injected to allow for human FcRn to interact with the antibody captured on the sensor chip. The running buffer used comprised 50 mmol/l sodium phosphate, 150 mmol/l NaCl, and 0.05% (w/v) Tween20 (pH 7.0). FcRn was diluted using each buffer. The chip was regenerated using 10 mmol/l glycine-HCl (pH 1.5). Assays were carried out at 25°C.

KD (M) of each antibody was derived from the sensorgram data using Biacore T100 Evaluation Software (GE Healthcare), which simultaneously fits the association and dissociation phases of the sensorgrams and globally fits all curves in the working set. Sensorgrams were fit to 1:1 binding model, the "Langmuir binding" model, supplied by Biacore T100 Evaluation Software. For some of the binding interactions, KD was derived by nonlinear regression analysis of plots of R_{eq}, the equilibrium binding response, versus the log of the analyte concentration using an equilibrium-based approach.

The result on the human FcRn binding under the neutral condition (pH 7.0) by Biacore is shown in Tables 27.

[Table 27]

	KD(M)
IgG1	8.8E-05
M73	1.4E-05
IgG1-v1	3.2E-06
IgG1-v2	8.1E-07

(9-3) *In vivo* studies of effect of antibodies on antigen elimination in co-administration model using human FcRn transgenic mouse line 276

In vivo study of antibodies using co-administration model was performed as described in Reference Example 3. Anti-human IL-6 receptor antibodies used in this study are the above-described H54/L28-IgG1, Fv4-IgG1, Fv4-M73, Fv4-IgG1-v1 and Fv4-IgG1-v2. Mice used in

this study is human FcRn transgenic mice (B6.mFcRn-/-.hFcRn Tg line 276 +/+ mouse, Jackson Laboratories; Methods Mol Biol. (2010) 602: 93-104).

As shown in Fig. 40, pharmacokinetics of H54/L28-IgG1, Fv4-IgG1, Fv4-M73, Fv4-IgG1-v1 and Fv4-IgG1-v2 were comparable, and these antibodies maintained similar plasma concentration during the study.

Time course of plasma hsIL-6R concentration was show in Fig. 41. Compared to the hsIL-6R administered with Fv4-IgG1, hsIL-6R administered with Fv4-IgG1-v2 exhibited enhanced clearance, whereas hsIL-6R administered with Fv4-M73 and Fv4-IgG1-v1 exhibited reduced clearance. Although all Fc variant, M73, v1, and v2 have increased binding affinity to human FcRn at neutral pH condition (pH 7.0), it was demonstrated that only Fv4-IgG1-v2, but not Fv4-M73 and Fv4-IgG1-v1, exhibited enhanced hsIL-6R clearance. This indicates that in order to enhance antigen clearance, binding affinity of antibody to human FcRn at pH 7.0 needs to be at least stronger than IgG1-v1, whose binding affinity to human FcRn at pH 7.0 is KD 3.2 μM or 28-fold stronger than intact human IgG1 (binding affinity to human FcRn is KD 88 μM).

Fig. 42 describes the relationship between the binding affinity of Fc variants to human FcRn at pH7.0 and plasma hsIL-6R concentration at day 1 after co-administration of hsIL-6R and Fc variants. Fc variants described in this Example and Reference Example 6 (Fv4-IgG1, Fv4-M73, Fv4-IgG1-v1, Fv4-IgG1-v2, Fv4-IgG1-F14, Fv4-IgG1-F20, Fv4-IgG1-F21, Fv4-IgG1-F25, Fv4-IgG1-F29, Fv4-IgG1-F35, Fv4-IgG1-F48, Fv4-IgG1-F93, and Fv4-IgG1-F94) are plotted. By increasing the binding affinity of antibody to human FcRn at pH 7.0, plasma concentration of hsIL-6R, which reflects the clearance of antigen, increased at first, but then decreased rapidly. This demonstrates that in order to enhance the antigen clearance compared to intact human IgG1, binding affinity of antibody to human FcRn at pH 7.0 needs to be preferably stronger than KD 2.3 μM (value obtained from curve fitting of Fig. 42). Binding affinity of antibody to human FcRn between KD 88 μM and KD 2.3 μM would rather reduce the antigen clearance (higher hsIL-6R concentration). In other words, binding affinity of antibody to human FcRn at pH 7.0 needs to be preferably 38-fold stronger than natural human IgG1 to enhance antigen elimination, or otherwise would reduce the antigen clearance.

Fig. 43 describes the relationship between the binding affinity of Fc variants to human FcRn at pH 7.0 and plasma antibody concentration at day 1 after co-administration of hsIL-6R and Fc variants. Fc variants described in this Example and Reference Example 6 (Fv4-IgG1, Fv4-M73, Fv4-IgG1-v1, Fv4-IgG1-v2, Fv4-IgG1-F14, Fv4-IgG1-F20, Fv4-IgG1-F21, Fv4-IgG1-F25, Fv4-IgG1-F29, Fv4-IgG1-F35, Fv4-IgG1-F48, Fv4-IgG1-F93, and Fv4-IgG1-F94) are plotted. By increasing the binding affinity of antibody to human FcRn at pH 7.0, plasma concentration of antibody, which reflects antibody pharmacokinetics (clearance), is maintained at first, but then decreased rapidly. This demonstrates that in order to maintain pharmacokinetics

of antibody similar to natural human IgG1 (binding affinity to human FcRn is KD 88 μM), affinity of antibody to human FcRn at pH 7.0 needs to be weaker than KD 0.2 μM (value obtained from curve fitting of Fig. 43). Binding affinity of antibody to human FcRn stronger than KD 0.2 μM increased the antibody clearance (i.e. more rapid antibody elimination from plasma). In other words, binding affinity of antibody to human FcRn at pH 7.0 needs to be within 440-fold stronger than natural human IgG1 to exhibit similar antibody pharmacokinetics as natural human IgG1, or otherwise would result in rapid antibody elimination from plasma.

Considering both Figs. 42 and 43, in order to enhance antigen clearance (*i.e.*, reduce antigen plasma concentration) compared to IgG1, while maintaining antibody pharmacokinetics similar to natural human IgG1, binding affinity of antibody to human FcRn at pH 7.0 needs to be between 2.3 μM and 0.2 μM, or in other words, binding affinity of antibody to human FcRn at pH 7.0 needs to be within a range of 38-fold to 440-fold stronger than intact human IgG1. Such antibody with similar pharmacokinetics as IgG1 with long-term antigen-elimination activity would be beneficial for antibody therapeutic which requires longer dosing interval such as chronic disease because of its long-acting property.

On the other hand, by increasing the binding affinity of antibody to human FcRn at pH 7.0 stronger than KD 0.2 μM, or in other words, by increasing the binding affinity of antibody to human FcRn at pH 7.0 more than 440-fold as compared to natural human IgG1, it would enhance antigen clearance to a large extent within a short-term, although antibody is eliminated from plasma faster than natural human IgG1. Such antibody with capability of inducing rapid and strong reduction of antigen concentration would be beneficial for antibody therapeutic such as acute disease in which disease related antigen needs to be removed from plasma because of its fast-acting property.

Amount of antigen eliminated from plasma per antibody is the important factor to evaluate the efficiency of antigen elimination by administrating the antibody Fc variants having increased binding affinity to human FcRn at pH 7.0. To evaluate the efficiency of antigen elimination per antibody, following calculation were conducted at each time point of *in vivo* study described in this Example and Reference Example 6.

value A: Molar antigen concentration at each time point

value B: Molar antibody concentration at each time point

value C: Molar antigen concentration per molar antibody concentration (molar antigen/antibody ratio) at each time point

$C = A/B$

Time courses of value C (molar antigen/antibody ratio) for each antibody were described in Fig. 44. Smaller value C indicates higher efficiency of antigen elimination per antibody whereas higher value C indicates lower efficiency of antigen elimination per antibody. Lower value C as compared to IgG1 indicates that higher antigen elimination efficiency was achieved by Fc variants, whereas higher value C as compared to IgG1 indicates that Fc variants have negative effect on antigen elimination efficiency. All the Fc variants except Fv4-M73 and Fv4-IgG1-v1 demonstrated enhanced antigen elimination efficiency as compared to Fv4-IgG1. Fv4-M73 and Fv4-IgG1-v1 demonstrated negative impact on antigen elimination efficiency, which was consistent with Fig. 42.

Fig. 45 describes the relationship between the binding affinity of Fc variants to human FcRn at pH 7.0 and value C (molar antigen/antibody ratio) at day 1 after co-administration of hsIL-6R and Fc variants. Fc variants described in this Example and Reference Example 6 (Fv4-IgG1, Fv4-M73, Fv4-IgG1-v1, Fv4-IgG1-v2, Fv4-IgG1-F14, Fv4-IgG1-F20, Fv4-IgG1-F21, Fv4-IgG1-F25, Fv4-IgG1-F29, Fv4-IgG1-F35, Fv4-IgG1-F48, Fv4-IgG1-F93, and Fv4-IgG1-F94) are plotted. This demonstrates that in order to achieve higher antigen elimination efficiency as compared to natural human IgG1, affinity of antibody to human FcRn at pH 7.0 needs to be stronger than KD 3.0 μM (value obtained from curve fitting of Fig. 45). In other words, binding affinity of antibody to human FcRn at pH 7.0 needs to be at least 29-fold stronger than natural human IgG1 to achieve higher antigen elimination efficiency as compared to natural human IgG1.

In conclusion, group of antibody variants having binding affinity to FcRn at pH 7.0 between KD 3.0 μM and 0.2 μM, or in other words, group of antibody variants having binding affinity to FcRn at pH 7.0 within a range of 29-fold to 440-fold stronger than natural human IgG1, have similar antibody pharmacokinetics to IgG1 but have enhanced capability to eliminate the antibody from plasma. Therefore, such antibody exhibits enhanced antigen elimination efficiency as compared to IgG1. Similar pharmacokinetics as IgG1 would enable long-term elimination of antigen from plasma (long-acting antigen elimination), and therefore long dosing intervals which would be preferable for antibody therapeutics for chronic disease. Group of antibody variants having binding affinity to FcRn at pH 7.0 stronger than KD 0.2 μM, or in other words, group of antibody variants having binding affinity to FcRn at pH 7.0 440-fold stronger than natural human IgG1, have rapid antibody clearance (short-term antibody elimination). Nevertheless, since such antibody enables even more rapid clearance of antigen (fast-acting antigen elimination), therefore, such antibody also exhibits enhanced antigen elimination efficiency as compared to IgG1. As shown in Reference Example 8, Fv4-IgG1-F20 in normal mouse would induce significant elimination of the antigen from plasma in a very short term, but the antigen elimination effect is not durable. Such profile would be preferable for acute diseases

where disease related antigen is needed to be depleted from plasma rapidly and significantly in a very short term.

[Reference Example 10] *In vivo* study of Fv4-IgG1-F14 by steady-state infusion model using human FcRn transgenic mouse line 276

In vivo study of Fv4-IgG1-F14 by steady-state infusion model using human FcRn transgenic mouse line 276 was performed as described below. Study group consists of control group (without antibody), Fv4-IgG1 at a dose of 1 mg/kg and Fv4-IgG1-F14 at a dose of 1 mg/kg, 0.2 mg/kg, and 0.01 mg/kg.

An infusion pump (MINI-OSMOTIC PUMP MODEL 2004; alzet) containing soluble human IL-6 receptor was implanted under the skin on the back of human FcRn transgenic mice 276 (B6.mFcRn-/-.hFcRn Tg line 276 +/+ mouse (B6.mFcRn-/- hFCRN Tg276 B6.Cg-Fcgrt <tm1Dcr> Tg(FCGRT) 276Dcr (Jackson #4919)), Jackson Laboratories; Methods Mol Biol. (2010) 602: 93-104) to prepare model animals where the plasma concentration of soluble human IL-6 receptor was kept constant. Anti-human IL-6 receptor antibodies were administered to the model animals to assess the *in vivo* dynamics after administration of soluble human IL-6 receptor. Monoclonal anti-mouse CD4 antibody (R&D) was administered at 20 mg/kg before implanting infusion pump and 14 days after antibody administration into the caudal vein to suppress the production of neutralizing antibody against soluble human IL-6 receptor. Then, an infusion pump containing 92.8 μg/ml soluble human IL-6 receptor was implanted under the skin on the back of the mice. Three days after implantation of an infusion pump, anti-human IL-6 receptor antibodies (H54/L28-IgG1 and H54/L28-IgG1-F14) were administered at 1 mg/kg once into the caudal vein. Blood was collected 15 minutes, seven hours, one day, two days, three days, four days, seven days, 14 days, 21 days, and 28 days after administration of the anti-human IL-6 receptor antibody. The collected blood was immediately centrifuged at 15,000 rpm and 4°C for 15 minutes to separate plasma. The separated plasma was stored in a refrigerator at -20°C or below before assay.

The concentration of hsIL-6R in mouse plasma was measured by electrochemiluminescence. hsIL-6R calibration curve samples adjusted to concentrations of 2,000, 1,000, 500, 250, 125, 62.5, and 31.25 pg/ml, and mouse plasma samples diluted 50-fold or more were prepared. The samples were mixed with a solution of Monoclonal Anti-human IL-6R Antibody (R&D) ruthenium-labeled with Sulfo-Tag NHS Ester (Meso Scale Discovery), Biotinylated Anti-human IL-6R Antibody (R&D), and WT-IgG1, and then allowed to react overnight at 37°C. The final concentration of WT-IgG1 as an anti-human IL-6 receptor antibody, comprising tocilizumab (heavy chain SEQ ID NO: 13; light chain SEQ ID NO: 14), was 333 μg/ml, which is in excess of the concentration of anti-human IL-6 receptor antibody contained in

the samples, for the purpose of binding nearly all of the hsIL-6R molecules in the samples to WT-IgG1. Subsequently, the samples were dispensed into an MA400 PR Streptavidin Plate (Meso Scale Discovery), and allowed to react for one hour at room temperature, and washing was performed. Immediately after Read Buffer T (x4) (Meso Scale Discovery) was dispensed, the measurement was performed by the Sector PR 400 Reader (Meso Scale Discovery). The hsIL-6R concentration was calculated based on the response of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices).

Fig. 46 describes time profile of hsIL-6R plasma concentration after antibody administration. Compared to baseline hsIL-6R level without antibody, administration of 1 mg/kg of Fv4-IgG1 resulted in several fold increase in plasma hsIL-6R concentration. On the other hands, administration of 1 mg/kg of Fv4-IgG1-F14 resulted in significant reduction in plasma concentration in comparison with Fv4-IgG1 group and baseline group. At day 2, plasma hsIL-6R concentration was not detected (quantitation limit of plasma hsIL-6R concentration is 1.56 ng/mL in this measurement system), and this lasted up to day 14.

H54/L28-IgG1-F14 exhibited reduction of plasma hsIL-6R concentration as compared to H54/L28-IgG1, but the extent of the reduction was small. Extent of reduction was much higher for Fv4 variable region which has pH dependent binding property to hsIL-6R. This demonstrates that although increasing binding affinity to human FcRn at pH 7.0 is effective for reducing plasma antigen concentration, combination of pH dependent antigen binding and increased binding affinity to human FcRn at neutral pH significantly enhances the antigen elimination.

Study using lower dose of Fv4-IgG1-F14 exhibited that even at 0.01 mg/kg, 1/100 of 1 mg/kg, reduced the antigen plasma concentration below the baseline demonstrating significant efficiency of the molecule to deplete the antigen from plasma.

[Reference Example 11] Comparison of human FcRn transgenic mouse lineage 276 and lineage 32 in co-administration model

Previous *in vivo* studies have been conducted using human FcRn transgenic mouse line 276 (Jackson Laboratories). In order to compare the difference between human FcRn transgenic mouse lineage 276 and a different transgenic line, lineage 32, we conducted co-administration study of H54/L28-IgG1, Fv4-IgG1, and Fv4-IgG1-v2 using human FcRn transgenic mouse lineage 32 (B6.mFcRn-/-.hFcRn Tg lineage 32 +/+ mouse (B6.mFcRn-/- hFCRN Tg32; B6.Cg-Fcgrt<tm1Dcr> Tg(FCGRT)32Dcr) (Jackson #4915)), Jackson Laboratories; Methods Mol Biol. (2010) 602: 93-104). Study method was same as that of Reference Example 3 but human FcRn transgenic mouse lineage 32 was used instead of human FcRn transgenic mouse lineage 276.

Fig. 47 describes the time course of plasma hsIL-6R concentration in both human FcRn transgenic mouse lineage 276 and lineage 32. H54/L28-IgG1, Fv4-IgG1, and Fv4-IgG1-v2 exhibited similar plasma hsIL-6R concentration time profile. In both mice, increasing binding affinity to human FcRn at pH 7.0 enhanced the antigen elimination from plasma (comparing Fv4-IgG1 and Fv4-IgG1-v2) to a same extent.

Fig. 48 describes the time course of plasma antibody concentration in both human FcRn transgenic mouse lineage 276 and lineage 32. H54/L28-IgG1, Fv4-IgG1, and Fv4-IgG1-v2 exhibited similar plasma antibody concentration time profile.

In conclusion, no significant difference were observed between lineage 276 and lineage 32, demonstrating that the Fc variant to increase the binding affinity to human FcRn at pH 7.0 was effective in two different transgenic mouse line expressing human FcRn for enhancing elimination of antigen plasma concentration.

[Reference Example 12] Generation of various antibody Fc variants having increased binding affinity to human FcRn at neutral pH

(12-1) Generation of Fc variants

Various mutations to increase the binding affinity to human FcRn under the neutral pH were introduced into Fv4-IgG1 to further improve the antigen elimination profile. Specifically, the amino acid mutations shown in Table 15, were introduced into the heavy chain constant region of Fv4-IgG1 to generate Fc variants (amino acid numbers of the mutation sites are described according to the EU numbering). The amino acid substitutions were introduced by the method known to those skilled in the art described in Reference Example 3.

The additional variants (IgG1-F100 to IgG1-F1052) each comprising a prepared heavy chain and L (WT) (SEQ ID NO: 14) were expressed and purified by methods known to those skilled in the art as described in Reference Example 3.

(12-2) Assessment of human FcRn binding

The binding between antibody and human FcRn was kinetically analyzed as described in Reference Example 5 for IgG1-v1, IgG1-v2 and IgG1-F2 to IgG1-F1052 or Reference Example 9 for IgG1 and M73. The result on the human FcRn binding under a neutral condition (pH 7.0) by Biacore is shown in Tables 28-1 to 28-21.

[Table 28-1]

VARIANT	KD (M)	AMINO ACID ALTERED POSITION
F1	8.10E-07	N434W
F2	3.20E-06	M252Y/S254T/T256E
F3	2.50E-06	N434Y
F4	5.80E-06	N434S
F5	6.80E-06	N434A
F7	5.60E-06	M252Y
F8	4.20E-06	M252W
F9	1.40E-07	M252Y/S254T/T256E/N434Y
F10	6.90E-08	M252Y/S254T/T256E/N434W
F11	3.10E-07	M252Y/N434Y
F12	1.70E-07	M252Y/N434W
F13	3.20E-07	M252W/N434Y
F14	1.80E-07	M252W/N434W
F19	4.60E-07	P257L/N434Y
F20	4.60E-07	V308F/N434Y
F21	3.00E-08	M252Y/V308P/N434Y
F22	2.00E-06	M428L/N434S
F25	9.20E-09	M252Y/S254T/T256E/V308P/N434W
F26	1.00E-06	I332V
F27	7.40E-06	G237M
F29	1.40E-06	I332V/N434Y
F31	2.80E-06	G237M/V308F
F32	8.00E-07	S254T/N434W
F33	2.30E-06	S254T/N434Y
F34	2.80E-07	T256E/N434W
F35	8.40E-07	T256E/N434Y
F36	3.60E-07	S254T/T256E/N434W
F37	1.10E-06	S254T/T256E/N434Y
F38	1.00E-07	M252Y/S254T/N434W
F39	3.00E-07	M252Y/S254T/N434Y
F40	8.20E-08	M252Y/T256E/N434W
F41	1.50E-07	M252Y/T256E/N434Y
F42	1.00E-06	M252Y/S254T/T256E/N434A
F43	1.70E-06	M252Y/N434A
F44	1.10E-06	M252W/N434A
F47	2.40E-07	M252Y/T256Q/N434W
F48	3.20E-07	M252Y/T256Q/N434Y
F49	5.10E-07	M252F/T256D/N434W
F50	1.20E-06	M252F/T256D/N434Y
F51	8.10E-06	N434F/Y436H

Table 28-2 is the continuation of Table 28-1.

[Table 28-2]

F52	3.10E-06	H433K/N434F/Y436H
F53	1.00E-06	I332V/N434W
F54	8.40E-08	V308P/N434W
F56	9.40E-07	I332V/M428L/N434Y
F57	1.10E-05	G385D/Q386P/N389S
F58	7.70E-07	G385D/Q386P/N389S/N434W
F59	2.40E-06	G385D/Q386P/N389S/N434Y
F60	1.10E-05	G385H
F61	9.70E-07	G385H/N434W
F62	1.90E-06	G385H/N434Y
F63	2.50E-06	N434F
F64	5.30E-06	N434H
F65	2.90E-07	M252Y/S254T/T256E/N434F
F66	4.30E-07	M252Y/S254T/T256E/N434H
F67	6.30E-07	M252Y/N434F
F68	9.30E-07	M252Y/N434H
F69	5.10E-07	M428L/N434W
F70	1.50E-06	M428L/N434Y
F71	8.30E-08	M252Y/S254T/T256E/M428L/N434W
F72	2.00E-07	M252Y/S254T/T256E/M428L/N434Y
F73	1.70E-07	M252Y/M428L/N434W
F74	4.60E-07	M252Y/M428L/N434Y
F75	1.40E-06	M252Y/M428L/N434A
F76	1.00E-06	M252Y/S254T/T256E/M428L/N434A
F77	9.90E-07	T256E/M428L/N434Y
F78	7.80E-07	S254T/M428L/N434W
F79	5.90E-06	S254T/T256E/N434A
F80	2.70E-06	M252Y/T256Q/N434A
F81	1.60E-06	M252Y/T256E/N434A
F82	1.10E-06	T256Q/N434W
F83	2.60E-06	T256Q/N434Y
F84	2.80E-07	M252W/T256Q/N434W
F85	5.50E-07	M252W/T256Q/N434Y
F86	1.50E-06	S254T/T256Q/N434W
F87	4.30E-06	S254T/T256Q/N434Y
F88	1.90E-07	M252Y/S254T/T256Q/N434W
F89	3.60E-07	M252Y/S254T/T256Q/N434Y
F90	1.90E-08	M252Y/T256E/V308P/N434W
F91	4.80E-08	M252Y/V308P/M428L/N434Y
F92	1.10E-08	M252Y/S254T/T256E/V308P/M428L/N434W
F93	7.40E-07	M252W/M428L/N434W
F94	3.70E-07	P257L/M428L/N434Y

Table 28-3 is the continuation of Table 28-2.

[Table 28-3]

F95	2.60E-07	M252Y/S254T/T256E/M428L/N434F
F99	6.20E-07	M252Y/T256E/N434H
F101	1.10E-07	M252W/T256Q/P257L/N434Y
F103	4.40E-08	P238A/M252Y/V308P/N434Y
F104	3.70E-08	M252Y/D265A/V308P/N434Y
F105	7.50E-08	M252Y/T307A/V308P/N434Y
F106	3.70E-08	M252Y/V303A/V308P/N434Y
F107	3.40E-08	M252Y/V308P/D376A/N434Y
F108	4.10E-08	M252Y/V305A/V308P/N434Y
F109	3.20E-08	M252Y/V308P/Q311A/N434Y
F111	3.20E-08	M252Y/V308P/K317A/N434Y
F112	6.40E-08	M252Y/V308P/E380A/N434Y
F113	3.20E-08	M252Y/V308P/E382A/N434Y
F114	3.80E-08	M252Y/V308P/S424A/N434Y
F115	6.60E-06	T307A/N434A
F116	8.70E-06	E380A/N434A
F118	1.40E-05	M428L
F119	5.40E-06	T250Q/M428L
F120	6.30E-08	P257L/V308P/M428L/N434Y
F121	1.50E-08	M252Y/T256E/V308P/M428L/N434W
F122	1.20E-07	M252Y/T256E/M428L/N434W
F123	3.00E-08	M252Y/T256E/V308P/N434Y
F124	2.90E-07	M252Y/T256E/M428L/N434Y
F125	2.40E-08	M252Y/S254T/T256E/V308P/M428L/N434Y
F128	1.70E-07	P257L/M428L/N434W
F129	2.20E-07	P257A/M428L/N434Y
F131	3.00E-06	P257G/M428L/N434Y
F132	2.10E-07	P257I/M428L/N434Y
F133	4.10E-07	P257M/M428L/N434Y
F134	2.70E-07	P257N/M428L/N434Y
F135	7.50E-07	P257S/M428L/N434Y
F136	3.80E-07	P257T/M428L/N434Y
F137	4.60E-07	P257V/M428L/N434Y
F139	1.50E-08	M252W/V308P/N434W
F140	3.60E-08	S239K/M252Y/V308P/N434Y
F141	3.50E-08	M252Y/S298G/V308P/N434Y
F142	3.70E-08	M252Y/D270F/V308P/N434Y
F143	2.00E-07	M252Y/V308A/N434Y
F145	5.30E-08	M252Y/V308F/N434Y
F147	2.40E-07	M252Y/V308I/N434Y
F149	1.90E-07	M252Y/V308L/N434Y
F150	2.00E-07	M252Y/V308M/N434Y

Table 28-4 is the continuation of Table 28-3.

[Table 28-4]

F152	2.70E-07	M252Y/V308Q/N434Y
F154	1.80E-07	M252Y/V308T/N434Y
F157	1.50E-07	P257A/V308P/M428L/N434Y
F158	5.90E-08	P257T/V308P/M428L/N434Y
F159	4.40E-08	P257V/V308P/M428L/N434Y
F160	8.50E-07	M252W/M428I/N434Y
F162	1.60E-07	M252W/M428Y/N434Y
F163	4.20E-07	M252W/M428F/N434Y
F164	3.70E-07	P238A/M252W/N434Y
F165	2.90E-07	M252W/D265A/N434Y
F166	1.50E-07	M252W/T307Q/N434Y
F167	2.90E-07	M252W/V303A/N434Y
F168	3.20E-07	M252W/D376A/N434Y
F169	2.90E-07	M252W/V305A/N434Y
F170	1.70E-07	M252W/Q311A/N434Y
F171	1.90E-07	M252W/D312A/N434Y
F172	2.20E-07	M252W/K317A/N434Y
F173	7.70E-07	M252W/E380A/N434Y
F174	3.40E-07	M252W/E382A/N434Y
F175	2.70E-07	M252W/S424A/N434Y
F176	2.90E-07	S239K/M252W/N434Y
F177	2.80E-07	M252W/S298G/N434Y
F178	2.70E-07	M252W/D270F/N434Y
F179	3.10E-07	M252W/N325G/N434Y
F182	6.60E-08	P257A/M428L/N434W
F183	2.20E-07	P257T/M428L/N434W
F184	2.70E-07	P257V/M428L/N434W
F185	2.60E-07	M252W/I332V/N434Y
F188	3.00E-06	P257I/Q311I
F189	1.90E-07	M252Y/T307A/N434Y
F190	1.10E-07	M252Y/T307Q/N434Y
F191	1.60E-07	P257L/T307A/M428L/N434Y
F192	1.10E-07	P257A/T307A/M428L/N434Y
F193	8.50E-08	P257T/T307A/M428L/N434Y
F194	1.20E-07	P257V/T307A/M428L/N434Y
F195	5.60E-08	P257L/T307Q/M428L/N434Y
F196	3.50E-08	P257A/T307Q/M428L/N434Y
F197	3.30E-08	P257T/T307Q/M428L/N434Y
F198	4.80E-08	P257V/T307Q/M428L/N434Y
F201	2.10E-07	M252Y/T307D/N434Y
F203	2.40E-07	M252Y/T307F/N434Y
F204	2.10E-07	M252Y/T307G/N434Y

Table 28-5 is the continuation of Table 28-4.

[Table 28-5]

F205	2.00E-07	M252Y/T307H/N434Y
F206	2.30E-07	M252Y/T307I/N434Y
F207	9.40E-07	M252Y/T307K/N434Y
F208	3.90E-07	M252Y/T307L/N434Y
F209	1.30E-07	M252Y/T307M/N434Y
F210	2.90E-07	M252Y/T307N/N434Y
F211	2.40E-07	M252Y/T307P/N434Y
F212	6.80E-07	M252Y/T307R/N434Y
F213	2.30E-07	M252Y/T307S/N434Y
F214	1.70E-07	M252Y/T307V/N434Y
F215	9.60E-08	M252Y/T307W/N434Y
F216	2.30E-07	M252Y/T307Y/N434Y
F217	2.30E-07	M252Y/K334L/N434Y
F218	2.60E-07	M252Y/G385H/N434Y
F219	2.50E-07	M252Y/T289H/N434Y
F220	2.50E-07	M252Y/Q311H/N434Y
F221	3.10E-07	M252Y/D312H/N434Y
F222	3.40E-07	M252Y/N315H/N434Y
F223	2.70E-07	M252Y/K360H/N434Y
F225	1.50E-06	M252Y/L314R/N434Y
F226	5.40E-07	M252Y/L314K/N434Y
F227	1.20E-07	M252Y/N286E/N434Y
F228	2.30E-07	M252Y/L309E/N434Y
F229	5.10E-07	M252Y/R255E/N434Y
F230	2.50E-07	M252Y/P387E/N434Y
F236	8.90E-07	K248I/M428L/N434Y
F237	2.30E-07	M252Y/M428A/N434Y
F238	7.40E-07	M252Y/M428D/N434Y
F240	7.20E-07	M252Y/M428F/N434Y
F241	1.50E-06	M252Y/M428G/N434Y
F242	8.50E-07	M252Y/M428H/N434Y
F243	1.80E-07	M252Y/M428I/N434Y
F244	1.30E-06	M252Y/M428K/N434Y
F245	4.70E-07	M252Y/M428N/N434Y
F246	1.10E-06	M252Y/M428P/N434Y
F247	4.40E-07	M252Y/M428Q/N434Y
F249	6.40E-07	M252Y/M428S/N434Y
F250	2.90E-07	M252Y/M428T/N434Y
F251	1.90E-07	M252Y/M428V/N434Y
F252	1.00E-06	M252Y/M428W/N434Y
F253	7.10E-07	M252Y/M428Y/N434Y
F254	7.50E-08	M252W/T307Q/M428Y/N434Y

Table 28-6 is the continuation of Table 28-5.

[Table 28-6]

F255	1.10E-07	M252W/Q311A/M428Y/N434Y
F256	5.40E-08	M252W/T307Q/Q311A/M428Y/N434Y
F257	5.00E-07	M252Y/T307A/M428Y/N434Y
F258	3.20E-07	M252Y/T307Q/M428Y/N434Y
F259	2.80E-07	M252Y/D270F/N434Y
F260	1.30E-07	M252Y/T307A/Q311A/N434Y
F261	8.40E-08	M252Y/T307Q/Q311A/N434Y
F262	1.90E-07	M252Y/T307A/Q311H/N434Y
F263	1.10E-07	M252Y/T307Q/Q311H/N434Y
F264	2.80E-07	M252Y/E382A/N434Y
F265	6.80E-07	M252Y/E382A/M428Y/N434Y
F266	4.70E-07	M252Y/T307A/E382A/M428Y/N434Y
F267	3.20E-07	M252Y/T307Q/E382A/M428Y/N434Y
F268	6.30E-07	P238A/M252Y/M428F/N434Y
F269	5.20E-07	M252Y/V305A/M428F/N434Y
F270	6.60E-07	M252Y/N325G/M428F/N434Y
F271	6.90E-07	M252Y/D376A/M428F/N434Y
F272	6.80E-07	M252Y/E380A/M428F/N434Y
F273	6.50E-07	M252Y/E382A/M428F/N434Y
F274	7.60E-07	M252Y/E380A/E382A/M428F/N434Y
F275	4.20E-08	S239K/M252Y/V308P/E382A/N434Y
F276	4.10E-08	M252Y/D270F/V308P/E382A/N434Y
F277	1.30E-07	S239K/M252Y/V308P/M428Y/N434Y
F278	3.00E-08	M252Y/T307Q/V308P/E382A/N434Y
F279	6.10E-08	M252Y/V308P/Q311H/E382A/N434Y
F280	4.10E-08	S239K/M252Y/D270F/V308P/N434Y
F281	9.20E-08	M252Y/V308P/E382A/M428F/N434Y
F282	2.90E-08	M252Y/V308P/E382A/M428L/N434Y
F283	1.00E-07	M252Y/V308P/E382A/M428Y/N434Y
F284	1.00E-07	M252Y/V308P/M428Y/N434Y
F285	9.90E-08	M252Y/V308P/M428F/N434Y
F286	1.20E-07	S239K/M252Y/V308P/E382A/M428Y/N434Y
F287	1.00E-07	M252Y/V308P/E380A/E382A/M428F/N434Y
F288	1.90E-07	M252Y/T256E/E382A/N434Y
F289	4.80E-07	M252Y/T256E/M428Y/N434Y
F290	4.60E-07	M252Y/T256E/E382A/M428Y/N434Y
F292	2.30E-08	S239K/M252Y/V308P/E382A/M428L/N434Y
F293	5.30E-08	M252Y/V308P/E380A/E382A/M428L/N434Y
F294	1.10E-07	S239K/M252Y/V308P/M428F/N434Y
F295	6.80E-07	S239K/M252Y/E380A/E382A/M428F/N434Y
F296	4.90E-07	M252Y/Q311A/M428Y/N434Y
F297	5.10E-07	M252Y/D312A/M428Y/N434Y

Table 28-7 is the continuation of Table 28-6.

[Table 28-7]

F298	4.80E-07	M252Y/Q311A/D312A/M428Y/N434Y
F299	9.40E-08	S239K/M252Y/V308P/Q311A/M428Y/N434Y
F300	8.30E-08	S239K/M252Y/V308P/D312A/M428Y/N434Y
F301	7.20E-08	S239K/M252Y/V308P/Q311A/D312A/M428Y/N434Y
F302	1.90E-07	M252Y/T256E/T307P/N434Y
F303	6.70E-07	M252Y/T307P/M428Y/N434Y
F304	1.60E-08	M252W/V308P/M428Y/N434Y
F305	2.70E-08	M252Y/T256E/V308P/E382A/N434Y
F306	3.60E-08	M252W/V308P/E382A/N434Y
F307	3.60E-08	S239K/M252W/V308P/E382A/N434Y
F308	1.90E-08	S239K/M252W/V308P/E382A/M428Y/N434Y
F310	9.40E-08	S239K/M252W/V308P/E382A/M428I/N434Y
F311	2.80E-08	S239K/M252W/V308P/M428F/N434Y
F312	4.50E-07	S239K/M252W/E380A/E382A/M428F/N434Y
F313	6.50E-07	S239K/M252Y/T307P/M428Y/N434Y
F314	3.20E-07	M252Y/T256E/Q311A/D312A/M428Y/N434Y
F315	6.80E-07	S239K/M252Y/M428Y/N434Y
F316	7.00E-07	S239K/M252Y/D270F/M428Y/N434Y
F317	1.10E-07	S239K/M252Y/D270F/V308P/M428Y/N434Y
F318	1.80E-08	S239K/M252Y/V308P/M428I/N434Y
F320	2.00E-08	S239K/M252Y/V308P/N325G/E382A/M428I/N434Y
F321	3.20E-08	S239K/M252Y/D270F/V308P/N325G/N434Y
F322	9.20E-08	S239K/M252Y/D270F/T307P/V308P/N434Y
F323	2.70E-08	S239K/M252Y/T256E/D270F/V308P/N434Y
F324	2.80E-08	S239K/M252Y/D270F/T307Q/V308P/N434Y
F325	2.10E-08	S239K/M252Y/D270F/T307Q/V308P/Q311A/N434Y
F326	7.50E-08	S239K/M252Y/D270F/T307Q/Q311A/N434Y
F327	6.50E-08	S239K/M252Y/T256E/D270F/T307Q/Q311A/N434Y
F328	1.90E-08	S239K/M252Y/D270F/V308P/M428I/N434Y
F329	1.20E-08	S239K/M252Y/D270F/N286E/V308P/N434Y
F330	3.60E-08	S239K/M252Y/D270F/V308P/L309E/N434Y
F331	3.00E-08	S239K/M252Y/D270F/V308P/P387E/N434Y
F333	7.40E-08	S239K/M252Y/D270F/T307Q/L309E/Q311A/N434Y
F334	1.90E-08	S239K/M252Y/D270F/V308P/N325G/M428I/N434Y
F335	1.50E-08	S239K/M252Y/T256E/D270F/V308P/M428I/N434Y
F336	1.40E-08	S239K/M252Y/D270F/T307Q/V308P/Q311A/M428I/N434Y
F337	5.60E-08	S239K/M252Y/D270F/T307Q/Q311A/M428I/N434Y
F338	7.70E-09	S239K/M252Y/D270F/N286E/V308P/M428I/N434Y
F339	1.90E-08	S239K/M252Y/D270F/V308P/L309E/M428I/N434Y
F343	3.20E-08	S239K/M252Y/D270F/V308P/M428L/N434Y
F344	3.00E-08	S239K/M252Y/V308P/M428L/N434Y
F349	1.50E-07	S239K/M252Y/V308P/L309P/M428L/N434Y

Table 28-8 is the continuation of Table 28-7.

[Table 28-8]

F350	1.70E-07	S239K/M252Y/V308P/L309R/M428L/N434Y
F352	6.00E-07	S239K/M252Y/L309P/M428L/N434Y
F353	1.10E-06	S239K/M252Y/L309R/M428L/N434Y
F354	2.80E-08	S239K/M252Y/T307Q/V308P/M428L/N434Y
F356	3.40E-08	S239K/M252Y/D270F/V308P/L309E/P387E/N434Y
F357	1.60E-08	S239K/M252Y/T256E/D270F/V308P/N325G/M428I/N434Y
F358	1.00E-07	S239K/M252Y/T307Q/N434Y
F359	4.20E-07	P257V/T307Q/M428I/N434Y
F360	1.30E-06	P257V/T307Q/M428V/N434Y
F362	5.40E-08	P257V/T307Q/N325G/M428L/N434Y
F363	4.10E-08	P257V/T307Q/Q311A/M428L/N434Y
F364	3.50E-08	P257V/T307Q/Q311A/N325G/M428L/N434Y
F365	5.10E-08	P257V/V305A/T307Q/M428L/N434Y
F367	1.50E-08	S239K/M252Y/E258H/D270F/T307Q/V308P/Q311A/N434Y
F368	2.00E-08	S239K/M252Y/D270F/V308P/N325G/E382A/M428I/N434Y
F369	7.50E-08	M252Y/P257V/T307Q/M428I/N434Y
F372	1.30E-08	S239K/M252W/V308P/M428Y/N434Y
F373	1.10E-08	S239K/M252W/V308P/Q311A/M428Y/N434Y
F374	1.20E-08	S239K/M252W/T256E/V308P/M428Y/N434Y
F375	5.50E-09	S239K/M252W/N286E/V308P/M428Y/N434Y
F376	9.60E-09	S239K/M252Y/T256E/D270F/N286E/V308P/N434Y
F377	1.30E-07	S239K/M252W/T307P/M428Y/N434Y
F379	9.00E-09	S239K/M252W/T256E/V308P/Q311A/M428Y/N434Y
F380	5.60E-09	S239K/M252W/T256E/N286E/V308P/M428Y/N434Y
F381	1.10E-07	P257V/T307A/Q311A/M428L/N434Y
F382	8.70E-08	P257V/V305A/T307A/M428L/N434Y
F386	3.20E-08	M252Y/V308P/L309E/N434Y
F387	1.50E-07	M252Y/V308P/L309D/N434Y
F388	7.00E-08	M252Y/V308P/L309A/N434Y
F389	1.70E-08	M252W/V308P/L309E/M428Y/N434Y
F390	6.80E-08	M252W/V308P/L309D/M428Y/N434Y
F391	3.60E-08	M252W/V308P/L309A/M428Y/N434Y
F392	6.90E-09	S239K/M252Y/N286E/V308P/M428I/N434Y
F393	1.20E-08	S239K/M252Y/N286E/V308P/N434Y
F394	5.30E-08	S239K/M252Y/T307Q/Q311A/M428I/N434Y
F395	2.40E-08	S239K/M252Y/T256E/V308P/N434Y
F396	2.00E-08	S239K/M252Y/D270F/N286E/T307Q/Q311A/M428I/N434Y
F397	4.50E-08	S239K/M252Y/D270F/T307Q/Q311A/P387E/M428I/N434Y
F398	4.40E-09	S239K/M252Y/D270F/N286E/T307Q/V308P/Q311A/M428I/N434Y
F399	6.50E-09	S239K/M252Y/D270F/N286E/T307Q/V308P/M428I/N434Y
F400	6.10E-09	S239K/M252Y/D270F/N286E/V308P/Q311A/M428I/N434Y

Table 28-9 is the continuation of Table 28-8.

[Table 28-9]

F401	6.90E-09	S239K/M252Y/D270F/N286E/V308P/P387E/M428I/N434Y
F402	2.30E-08	P257V/T307Q/M428L/N434W
F403	5.10E-08	P257V/T307A/M428L/N434W
F404	9.40E-08	P257A/T307Q/L309P/M428L/N434Y
F405	1.70E-07	P257V/T307Q/L309P/M428L/N434Y
F406	1.50E-07	P257A/T307Q/L309R/M428L/N434Y
F407	1.60E-07	P257V/T307Q/L309R/M428L/N434Y
F408	2.50E-07	P257V/N286E/M428L/N434Y
F409	2.00E-07	P257V/P387E/M428L/N434Y
F410	2.20E-07	P257V/T307H/M428L/N434Y
F411	1.30E-07	P257V/T307N/M428L/N434Y
F412	8.80E-08	P257V/T307G/M428L/N434Y
F413	1.20E-07	P257V/T307P/M428L/N434Y
F414	1.10E-07	P257V/T307S/M428L/N434Y
F415	5.60E-08	P257V/N286E/T307A/M428L/N434Y
F416	9.40E-08	P257V/T307A/P387E/M428L/N434Y
F418	6.20E-07	S239K/M252Y/T307P/N325G/M428Y/N434Y
F419	1.60E-07	M252Y/T307A/Q311H/K360H/N434Y
F420	1.50E-07	M252Y/T307A/Q311H/P387E/N434Y
F421	1.30E-07	M252Y/T307A/Q311H/M428A/N434Y
F422	1.80E-07	M252Y/T307A/Q311H/E382A/N434Y
F423	8.40E-08	M252Y/T307W/Q311H/N434Y
F424	9.40E-08	S239K/P257A/V308P/M428L/N434Y
F425	8.00E-08	P257A/V308P/L309E/M428L/N434Y
F426	8.40E-08	P257V/T307Q/N434Y
F427	1.10E-07	M252Y/P257V/T307Q/M428V/N434Y
F428	8.00E-08	M252Y/P257V/T307Q/M428L/N434Y
F429	3.70E-08	M252Y/P257V/T307Q/N434Y
F430	8.10E-08	M252Y/P257V/T307Q/M428Y/N434Y
F431	6.50E-08	M252Y/P257V/T307Q/M428F/N434Y
F432	9.20E-07	P257V/T307Q/Q311A/N325G/M428V/N434Y
F433	6.00E-08	P257V/T307Q/Q311A/N325G/N434Y
F434	2.00E-08	P257V/T307Q/Q311A/N325G/M428Y/N434Y
F435	2.50E-08	P257V/T307Q/Q311A/N325G/M428F/N434Y
F436	2.50E-07	P257A/T307Q/M428V/N434Y
F437	5.70E-08	P257A/T307Q/N434Y
F438	3.60E-08	P257A/T307Q/M428Y/N434Y
F439	4.00E-08	P257A/T307Q/M428F/N434Y
F440	1.50E-08	P257V/N286E/T307Q/Q311A/N325G/M428L/N434Y
F441	1.80E-07	P257A/Q311A/M428L/N434Y
F442	2.00E-07	P257A/Q311H/M428L/N434Y
F443	5.50E-08	P257A/T307Q/Q311A/M428L/N434Y

Table 28-10 is the continuation of Table 28-9.

[Table 28-10]

F444	1.40E-07	P257A/T307A/Q311A/M428L/N434Y
F445	6.20E-08	P257A/T307Q/Q311H/M428L/N434Y
F446	1.10E-07	P257A/T307A/Q311H/M428L/N434Y
F447	1.40E-08	P257A/N286E/T307Q/M428L/N434Y
F448	5.30E-08	P257A/N286E/T307A/M428L/N434Y
F449	5.70E-07	S239K/M252Y/D270F/T307P/N325G/M428Y/N434Y
F450	5.20E-07	S239K/M252Y/T307P/L309E/N325G/M428Y/N434Y
F451	1.00E-07	P257S/T307A/M428L/N434Y
F452	1.40E-07	P257M/T307A/M428L/N434Y
F453	7.80E-08	P257N/T307A/M428L/N434Y
F454	9.60E-08	P257I/T307A/M428L/N434Y
F455	2.70E-08	P257V/T307Q/M428Y/N434Y
F456	3.40E-08	P257V/T307Q/M428F/N434Y
F457	4.00E-08	S239K/P257V/V308P/M428L/N434Y
F458	1.50E-08	P257V/T307Q/V308P/N325G/M428L/N434Y
F459	1.30E-08	P257V/T307Q/V308P/Q311A/N325G/M428L/N434Y
F460	4.70E-08	P257V/T307A/V308P/N325G/M428L/N434Y
F462	8.50E-08	P257A/V308P/N325G/M428L/N434Y
F463	1.30E-07	P257A/T307A/V308P/M428L/N434Y
F464	5.50E-08	P257A/T307Q/V308P/M428L/N434Y
F465	2.10E-08	P257V/N286E/T307Q/N325G/M428L/N434Y
F466	3.50E-07	T256E/P257V/N434Y
F467	5.70E-07	T256E/P257T/N434Y
F468	5.70E-08	S239K/P257T/V308P/M428L/N434Y
F469	5.60E-08	P257T/V308P/N325G/M428L/N434Y
F470	5.40E-08	T256E/P257T/V308P/N325G/M428L/N434Y
F471	6.60E-08	P257T/V308P/N325G/E382A/M428L/N434Y
F472	5.40E-08	P257T/V308P/N325G/P387E/M428L/N434Y
F473	4.50E-07	P257T/V308P/L309P/N325G/M428L/N434Y
F474	3.50E-07	P257T/V308P/L309R/N325G/M428L/N434Y
F475	4.30E-08	T256E/P257V/T307Q/M428L/N434Y
F476	5.50E-08	P257V/T307Q/E382A/M428L/N434Y
F477	4.30E-08	P257V/T307Q/P387E/M428L/N434Y
F480	3.90E-08	P257L/V308P/N434Y
F481	5.60E-08	P257T/T307Q/N434Y
F482	7.00E-08	P257V/T307Q/N325G/N434Y
F483	5.70E-08	P257V/T307Q/Q311A/N434Y
F484	6.20E-08	P257V/V305A/T307Q/N434Y
F485	9.70E-08	P257V/N286E/T307A/N434Y
F486	3.40E-07	P257V/T307Q/L309R/Q311H/M428L/N434Y
F488	3.50E-08	P257V/V308P/N325G/M428L/N434Y
F490	7.50E-08	S239K/P257V/V308P/Q311H/M428L/N434Y

Table 28-11 is the continuation of Table 28-10.

[Table 28-11]

F492	9.80E-08	P257V/V305A/T307A/N325G/M428L/N434Y
F493	4.90E-07	S239K/D270F/T307P/N325G/M428Y/N434Y
F497	3.10E-06	P257T/T307A/M428V/N434Y
F498	1.30E-06	P257A/M428V/N434Y
F499	5.20E-07	P257A/T307A/M428V/N434Y
F500	4.30E-08	P257S/T307Q/M428L/N434Y
F506	1.90E-07	P257V/N297A/T307Q/M428L/N434Y
F507	5.10E-08	P257V/N286A/T307Q/M428L/N434Y
F508	1.10E-07	P257V/T307Q/N315A/M428L/N434Y
F509	5.80E-08	P257V/T307Q/N384A/M428L/N434Y
F510	5.30E-08	P257V/T307Q/N389A/M428L/N434Y
F511	4.20E-07	P257V/N434Y
F512	5.80E-07	P257T/N434Y
F517	3.10E-07	P257V/N286E/N434Y
F518	4.20E-07	P257T/N286E/N434Y
F519	2.60E-08	P257V/N286E/T307Q/N434Y
F521	1.10E-08	P257V/N286E/T307Q/M428Y/N434Y
F523	2.60E-08	P257V/V305A/T307Q/M428Y/N434Y
F526	1.90E-08	P257T/T307Q/M428Y/N434Y
F527	9.40E-09	P257V/T307Q/V308P/N325G/M428Y/N434Y
F529	2.50E-08	P257T/T307Q/M428F/N434Y
F533	1.20E-08	P257A/N286E/T307Q/M428F/N434Y
F534	1.20E-08	P257A/N286E/T307Q/M428Y/N434Y
F535	3.90E-08	T250A/P257V/T307Q/M428L/N434Y
F538	9.90E-08	T250F/P257V/T307Q/M428L/N434Y
F541	6.00E-08	T250I/P257V/T307Q/M428L/N434Y
F544	3.10E-08	T250M/P257V/T307Q/M428L/N434Y
F549	5.40E-08	T250S/P257V/T307Q/M428L/N434Y
F550	5.90E-08	T250V/P257V/T307Q/M428L/N434Y
F551	1.20E-07	T250W/P257V/T307Q/M428L/N434Y
F552	1.10E-07	T250Y/P257V/T307Q/M428L/N434Y
F553	1.70E-07	M252Y/Q311A/N434Y
F554	2.80E-08	S239K/M252Y/S254T/V308P/N434Y
F556	1.50E-06	M252Y/T307Q/Q311A
F559	8.00E-08	M252Y/S254T/N286E/N434Y
F560	2.80E-08	M252Y/S254T/V308P/N434Y
F561	1.40E-07	M252Y/S254T/T307A/N434Y
F562	8.30E-08	M252Y/S254T/T307Q/N434Y
F563	1.30E-07	M252Y/S254T/Q311A/N434Y
F564	1.90E-07	M252Y/S254T/Q311H/N434Y
F565	9.20E-08	M252Y/S254T/T307A/Q311A/N434Y
F566	6.10E-08	M252Y/S254T/T307Q/Q311A/N434Y

Table 28-12 is the continuation of Table 28-11.

[Table 28-12]

F567	2.20E-07	M252Y/S254T/M428I/N434Y
F568	1.10E-07	M252Y/T256E/T307A/Q311H/N434Y
F569	2.00E-07	M252Y/T256Q/T307A/Q311H/N434Y
F570	1.30E-07	M252Y/S254T/T307A/Q311H/N434Y
F571	8.10E-08	M252Y/N286E/T307A/Q311H/N434Y
F572	1.00E-07	M252Y/T307A/Q311H/M428L/N434Y
F576	1.60E-06	M252Y/T256E/T307Q/Q311H
F577	1.30E-06	M252Y/N286E/T307A/Q311A
F578	5.70E-07	M252Y/N286E/T307Q/Q311A
F580	8.60E-07	M252Y/N286E/T307Q/Q311H
F581	7.20E-08	M252Y/T256E/N286E/N434Y
F582	7.50E-07	S239K/M252Y/V308P
F583	7.80E-07	S239K/M252Y/V308P/E382A
F584	6.30E-07	S239K/M252Y/T256E/V308P
F585	2.90E-07	S239K/M252Y/N286E/V308P
F586	1.40E-07	S239K/M252Y/N286E/V308P/M428I
F587	1.90E-07	M252Y/N286E/M428L/N434Y
F592	2.00E-07	M252Y/S254T/E382A/N434Y
F593	3.10E-08	S239K/M252Y/S254T/V308P/M428I/N434Y
F594	1.60E-08	S239K/M252Y/T256E/V308P/M428I/N434Y
F595	1.80E-07	S239K/M252Y/M428I/N434Y
F596	4.00E-07	M252Y/D312A/E382A/M428Y/N434Y
F597	2.20E-07	M252Y/E382A/P387E/N434Y
F598	1.40E-07	M252Y/D312A/P387E/N434Y
F599	5.20E-07	M252Y/P387E/M428Y/N434Y
F600	2.80E-07	M252Y/T256Q/E382A/N434Y
F601	9.60E-09	M252Y/N286E/V308P/N434Y
F608		G236A/S239D/I332E
F611	2.80E-07	M252Y/V305T/T307P/V308I/L309A/N434Y
F612	3.60E-07	M252Y/T307P/V308I/L309A/N434Y
F613		S239D/A330L/I332E
F616		S239D/K326D/L328Y
F617	7.40E-07	S239K/N434W
F618	6.40E-07	S239K/V308F/N434Y
F619	3.10E-07	S239K/M252Y/N434Y
F620	2.10E-07	S239K/M252Y/S254T/N434Y
F621	1.50E-07	S239K/M252Y/T307A/Q311H/N434Y
F622	3.50E-07	S239K/M252Y/T256Q/N434Y
F623	1.80E-07	S239K/M252W/N434W
F624	1.40E-08	S239K/P257A/N286E/T307Q/M428L/N434Y
F625	7.60E-08	S239K/P257A/T307Q/M428L/N434Y
F626	1.30E-06	V308P

Table 28-13 is the continuation of Table 28-12.

[Table 28-13]

F629	3.90E-08	M252Y/V279L/V308P/N434Y
F630	3.70E-08	S239K/M252Y/V279L/V308P/N434Y
F633	2.40E-08	M252Y/V282D/V308P/N434Y
F634	3.20E-08	S239K/M252Y/V282D/V308P/N434Y
F635	4.50E-08	M252Y/V284K/V308P/N434Y
F636	4.80E-08	S239K/M252Y/V284K/V308P/N434Y
F637	1.50E-07	M252Y/K288S/V308P/N434Y
F638	1.40E-07	S239K/M252Y/K288S/V308P/N434Y
F639	2.70E-08	M252Y/V308P/G385R/N434Y
F640	3.60E-08	S239K/M252Y/V308P/G385R/N434Y
F641	3.00E-08	M252Y/V308P/Q386K/N434Y
F642	3.00E-08	S239K/M252Y/V308P/Q386K/N434Y
F643	3.20E-08	L235G/G236R/S239K/M252Y/V308P/N434Y
F644	3.00E-08	G236R/S239K/M252Y/V308P/N434Y
F645	3.30E-08	S239K/M252Y/V308P/L328R/N434Y
F646	3.80E-08	S239K/M252Y/N297A/V308P/N434Y
F647	2.90E-08	P238D/M252Y/V308P/N434Y
F648		P238D
F649	1.20E-07	S239K/M252Y/N286E/N434Y
F650	1.70E-07	S239K/M252Y/T256E/N434Y
F651	1.80E-07	S239K/M252Y/Q311A/N434Y
F652	2.40E-07	P238D/M252Y/N434Y
F654	3.20E-08	L235K/S239K/M252Y/V308P/N434Y
F655	3.40E-08	L235R/S239K/M252Y/V308P/N434Y
F656	3.30E-08	G237K/S239K/M252Y/V308P/N434Y
F657	3.20E-08	G237R/S239K/M252Y/V308P/N434Y
F658	3.20E-08	P238K/S239K/M252Y/V308P/N434Y
F659	3.00E-08	P238R/S239K/M252Y/V308P/N434Y
F660	3.10E-08	S239K/M252Y/V308P/P329K/N434Y
F661	3.40E-08	S239K/M252Y/V308P/P329R/N434Y
F663	6.40E-09	S239K/M252Y/N286E/T307Q/V308P/Q311A/N434Y
F664	3.90E-08	M252Y/N286A/V308P/N434Y
F665	2.00E-08	M252Y/N286D/V308P/N434Y
F666	2.10E-08	M252Y/N286F/V308P/N434Y
F667	3.00E-08	M252Y/N286G/V308P/N434Y
F668	4.00E-08	M252Y/N286H/V308P/N434Y
F669	3.50E-08	M252Y/N286I/V308P/N434Y
F670	2.10E-07	M252Y/N286K/V308P/N434Y
F671	2.20E-08	M252Y/N286L/V308P/N434Y
F672	2.40E-08	M252Y/N286M/V308P/N434Y
F673	2.30E-08	M252Y/N286P/V308P/N434Y
F674	3.20E-08	M252Y/N286Q/V308P/N434Y

Table 28-14 is the continuation of Table 28-13.

[Table 28-14]

F675	5.10E-08	M252Y/N286R/V308P/N434Y
F676	3.20E-08	M252Y/N286S/V308P/N434Y
F677	4.70E-08	M252Y/N286T/V308P/N434Y
F678	3.30E-08	M252Y/N286V/V308P/N434Y
F679	1.70E-08	M252Y/N286W/V308P/N434Y
F680	1.50E-08	M252Y/N286Y/V308P/N434Y
F681	4.90E-08	M252Y/K288A/V308P/N434Y
F682	8.20E-08	M252Y/K288D/V308P/N434Y
F683	5.00E-08	M252Y/K288E/V308P/N434Y
F684	5.10E-08	M252Y/K288F/V308P/N434Y
F685	5.30E-08	M252Y/K288G/V308P/N434Y
F686	4.60E-08	M252Y/K288H/V308P/N434Y
F687	4.90E-08	M252Y/K288I/V308P/N434Y
F688	2.80E-08	M252Y/K288L/V308P/N434Y
F689	4.10E-08	M252Y/K288M/V308P/N434Y
F690	1.00E-07	M252Y/K288N/V308P/N434Y
F691	3.20E-07	M252Y/K288P/V308P/N434Y
F692	3.90E-08	M252Y/K288Q/V308P/N434Y
F693	3.60E-08	M252Y/K288R/V308P/N434Y
F694	4.70E-08	M252Y/K288V/V308P/N434Y
F695	4.00E-08	M252Y/K288W/V308P/N434Y
F696	4.40E-08	M252Y/K288Y/V308P/N434Y
F697	3.10E-08	S239K/M252Y/V308P/N325G/N434Y
F698	2.20E-08	M252Y/N286E/T307Q/Q311A/N434Y
F699	2.30E-08	S239K/M252Y/N286E/T307Q/Q311A/N434Y
F700	5.20E-08	M252Y/V308P/L328E/N434Y
F705	7.10E-09	M252Y/N286E/V308P/M428I/N434Y
F706	1.80E-08	M252Y/N286E/T307Q/Q311A/M428I/N434Y
F707	5.90E-09	M252Y/N286E/T307Q/V308P/Q311A/N434Y
F708	4.10E-09	M252Y/N286E/T307Q/V308P/Q311A/M428I/N434Y
F709	2.00E-08	S239K/M252Y/N286E/T307Q/Q311A/M428I/N434Y
F710	1.50E-08	P238D/M252Y/N286E/T307Q/Q311A/M428I/N434Y
F711	6.50E-08	S239K/M252Y/T307Q/Q311A/N434Y
F712	6.00E-08	P238D/M252Y/T307Q/Q311A/N434Y
F713	2.00E-08	P238D/M252Y/N286E/T307Q/Q311A/N434Y
F714	2.30E-07	P238D/M252Y/N325S/N434Y
F715	2.30E-07	P238D/M252Y/N325M/N434Y
F716	2.70E-07	P238D/M252Y/N325L/N434Y
F717	2.60E-07	P238D/M252Y/N325I/N434Y
F718	2.80E-07	P238D/M252Y/Q295M/N434Y
F719	7.40E-08	P238D/M252Y/N325G/N434Y
F720	2.40E-08	M252Y/T307Q/V308P/Q311A/N434Y

Table 28-15 is the continuation of Table 28-14.

[Table 28-15]

F721	1.50E-08	M252Y/T307Q/V308P/Q311A/M428I/N434Y
F722	2.70E-07	P238D/M252Y/A327G/N434Y
F723	2.80E-07	P238D/M252Y/L328D/N434Y
F724	2.50E-07	P238D/M252Y/L328E/N434Y
F725	4.20E-08	L235K/G237R/S239K/M252Y/V308P/N434Y
F726	3.70E-08	L235K/P238K/S239K/M252Y/V308P/N434Y
F729	9.20E-07	T307A/Q311A/N434Y
F730	6.00E-07	T307Q/Q311A/N434Y
F731	8.50E-07	T307A/Q311H/N434Y
F732	6.80E-07	T307Q/Q311H/N434Y
F733	3.20E-07	M252Y/L328E/N434Y
F734	3.10E-07	G236D/M252Y/L328E/N434Y
F736	3.10E-07	M252Y/S267M/L328E/N434Y
F737	3.10E-07	M252Y/S267L/L328E/N434Y
F738	3.50E-07	P238D/M252Y/T307P/N434Y
F739	2.20E-07	M252Y/T307P/Q311A/N434Y
F740	2.90E-07	M252Y/T307P/Q311H/N434Y
F741	3.10E-07	P238D/T250A/M252Y/N434Y
F744	9.90E-07	P238D/T250F/M252Y/N434Y
F745	6.60E-07	P238D/T250G/M252Y/N434Y
F746	6.00E-07	P238D/T250H/M252Y/N434Y
F747	2.80E-07	P238D/T250I/M252Y/N434Y
F749	5.10E-07	P238D/T250L/M252Y/N434Y
F750	3.00E-07	P238D/T250M/M252Y/N434Y
F751	5.30E-07	P238D/T250N/M252Y/N434Y
F753	1.80E-07	P238D/T250Q/M252Y/N434Y
F755	3.50E-07	P238D/T250S/M252Y/N434Y
F756	3.70E-07	P238D/T250V/M252Y/N434Y
F757	1.20E-06	P238D/T250W/M252Y/N434Y
F758	1.40E-06	P238D/T250Y/M252Y/N434Y
F759		L235K/S239K
F760		L235R/S239K
F761	1.10E-06	P238D/N434Y
F762	3.60E-08	L235K/S239K/M252Y/N286E/T307Q/Q311A/N434Y
F763	3.50E-08	L235R/S239K/M252Y/N286E/T307Q/Q311A/N434Y
F764	6.30E-07	P238D/T307Q/Q311A/N434Y
F765	8.50E-08	P238D/M252Y/T307Q/L309E/Q311A/N434Y
F766	6.00E-07	T307A/L309E/Q311A/N434Y
F767	4.30E-07	T307Q/L309E/Q311A/N434Y
F768	6.40E-07	T307A/L309E/Q311H/N434Y
F769	4.60E-07	T307Q/L309E/Q311H/N434Y
F770	3.00E-07	M252Y/T256A/N434Y

Table 28-16 is the continuation of Table 28-15.

[Table 28-16]

F771	4.00E-07	M252Y/E272A/N434Y
F772	3.80E-07	M252Y/K274A/N434Y
F773	3.90E-07	M252Y/V282A/N434Y
F774	4.00E-07	M252Y/N286A/N434Y
F775	6.20E-07	M252Y/K338A/N434Y
F776	3.90E-07	M252Y/K340A/N434Y
F777	3.90E-07	M252Y/E345A/N434Y
F779	3.90E-07	M252Y/N361A/N434Y
F780	3.90E-07	M252Y/Q362A/N434Y
F781	3.70E-07	M252Y/S375A/N434Y
F782	3.50E-07	M252Y/Y391A/N434Y
F783	4.00E-07	M252Y/D413A/N434Y
F784	5.00E-07	M252Y/L309A/N434Y
F786	7.40E-07	M252Y/L309H/N434Y
F786	2.80E-08	M252Y/S254T/N286E/T307Q/Q311A/N434Y
F787	8.80E-08	M252Y/S254T/T307Q/L309E/Q311A/N434Y
F788	4.10E-07	M252Y/N315A/N434Y
F789	1.50E-07	M252Y/N315D/N434Y
F790	2.70E-07	M252Y/N315E/N434Y
F791	4.40E-07	M252Y/N315F/N434Y
F792	4.40E-07	M252Y/N315G/N434Y
F793	3.30E-07	M252Y/N315I/N434Y
F794	4.10E-07	M252Y/N315K/N434Y
F795	3.10E-07	M252Y/N315L/N434Y
F796	3.40E-07	M252Y/N315M/N434Y
F798	3.50E-07	M252Y/N315Q/N434Y
F799	4.10E-07	M252Y/N315R/N434Y
F800	3.80E-07	M252Y/N315S/N434Y
F801	4.40E-07	M252Y/N315T/N434Y
F802	3.30E-07	M252Y/N315V/N434Y
F803	3.60E-07	M252Y/N315W/N434Y
F804	4.00E-07	M252Y/N315Y/N434Y
F805	3.00E-07	M252Y/N325A/N434Y
F806	3.10E-07	M252Y/N384A/N434Y
F807	3.20E-07	M252Y/N389A/N434Y
F808	3.20E-07	M252Y/N389A/N390A/N434Y
F809	2.20E-07	M252Y/S254T/T256S/N434Y
F810	2.20E-07	M252Y/A378V/N434Y
F811	4.90E-07	M252Y/E380S/N434Y
F812	2.70E-07	M252Y/E382V/N434Y
F813	2.80E-07	M252Y/S424E/N434Y
F814	1.20E-07	M252Y/N434Y/Y436I

Table 28-17 is the continuation of Table 28-16.

[Table 28-17]

F815	5.50E-07	M252Y/N434Y/T437R
F816	3.60E-07	P238D/T250V/M252Y/T307P/N434Y
F817	9.80E-08	P238D/T250V/M252Y/T307Q/Q311A/N434Y
F819	1.40E-07	P238D/M252Y/N286E/N434Y
F820	3.40E-07	L235K/S239K/M252Y/N434Y
F821	3.10E-07	L235R/S239K/M252Y/N434Y
F822	1.10E-06	P238D/T250Y/M252Y/W313Y/N434Y
F823	1.10E-06	P238D/T250Y/M252Y/W313F/N434Y
F828	2.50E-06	P238D/T250V/M252Y/I253V/N434Y
F831	1.60E-06	P238D/T250V/M252Y/R255A/N434Y
F832	2.60E-06	P238D/T250V/M252Y/R255D/N434Y
F833	8.00E-07	P238D/T250V/M252Y/R255E/N434Y
F834	8.10E-07	P238D/T250V/M252Y/R255F/N434Y
F836	5.00E-07	P238D/T250V/M252Y/R255H/N434Y
F837	5.60E-07	P238D/T250V/M252Y/R255I/N434Y
F838	4.30E-07	P238D/T250V/M252Y/R255K/N434Y
F839	3.40E-07	P238D/T250V/M252Y/R255L/N434Y
F840	4.20E-07	P238D/T250V/M252Y/R255M/N434Y
F841	1.10E-06	P238D/T250V/M252Y/R255N/N434Y
F843	6.60E-07	P238D/T250V/M252Y/R255Q/N434Y
F844	1.30E-06	P238D/T250V/M252Y/R255S/N434Y
F847	3.40E-07	P238D/T250V/M252Y/R255W/N434Y
F848	8.30E-07	P238D/T250V/M252Y/R255Y/N434Y
F849	3.30E-07	M252Y/D280A/N434Y
F850	2.90E-07	M252Y/D280E/N434Y
F852	3.30E-07	M252Y/D280G/N434Y
F853	3.20E-07	M252Y/D280H/N434Y
F855	3.20E-07	M252Y/D280K/N434Y
F858	3.20E-07	M252Y/D280N/N434Y
F860	3.30E-07	M252Y/D280Q/N434Y
F861	3.20E-07	M252Y/D280R/N434Y
F862	3.00E-07	M252Y/D280S/N434Y
F863	2.70E-07	M252Y/D280T/N434Y
F867	2.80E-07	M252Y/N384A/N389A/N434Y
F868	2.00E-08	G236A/S239D/M252Y/N286E/T307Q/Q311A/N434Y
F869		G236A/S239D
F870	7.30E-08	L235K/S239K/M252Y/T307Q/Q311A/N434Y
F871	7.10E-08	L235R/S239K/M252Y/T307Q/Q311A/N434Y
F872	1.30E-07	L235K/S239K/M252Y/N286E/N434Y
F873	1.20E-07	L235R/S239K/M252Y/N286E/N434Y
F875	4.80E-07	M252Y/N434Y/Y436A
F877	8.30E-07	M252Y/N434Y/Y436E

Table 28-18 is the continuation of Table 28-17.

[Table 28-18]

F878	1.90E-07	M252Y/N434Y/Y436F
F879	9.20E-07	M252Y/N434Y/Y436G
F880	3.90E-07	M252Y/N434Y/Y436H
F881	3.10E-07	M252Y/N434Y/Y436K
F882	1.30E-07	M252Y/N434Y/Y436L
F883	2.10E-07	M252Y/N434Y/Y436M
F884	4.00E-07	M252Y/N434Y/Y436N
F888	4.80E-07	M252Y/N434Y/Y436S
F889	2.20E-07	M252Y/N434Y/Y436T
F890	1.10E-07	M252Y/N434Y/Y436V
F891	1.70E-07	M252Y/N434Y/Y436W
F892	7.10E-08	M252Y/S254T/N434Y/Y436I
F893	9.80E-08	L235K/S239K/M252Y/N434Y/Y436I
F894	9.20E-08	L235R/S239K/M252Y/N434Y/Y436I
F895	2.10E-08	L235K/S239K/M252Y/N286E/T307Q/Q311A/N315E/N434Y
F896	2.00E-08	L235R/S239K/M252Y/N286E/T307Q/Q311A/N315E/N434Y
F897	9.70E-08	M252Y/N315D/N384A/N389A/N434Y
F898	1.70E-07	M252Y/N315E/N384A/N389A/N434Y
F899	1.10E-07	M252Y/N315D/G316A/N434Y
F900	1.70E-07	M252Y/N315D/G316D/N434Y
F901	1.30E-07	M252Y/N315D/G316E/N434Y
F902	2.20E-07	M252Y/N315D/G316F/N434Y
F903	2.30E-07	M252Y/N315D/G316H/N434Y
F904	1.00E-07	M252Y/N315D/G316I/N434Y
F905	1.30E-07	M252Y/N315D/G316K/N434Y
F906	1.50E-07	M252Y/N315D/G316L/N434Y
F907	1.30E-07	M252Y/N315D/G316M/N434Y
F908	1.50E-07	M252Y/N315D/G316N/N434Y
F909	1.30E-07	M252Y/N315D/G316P/N434Y
F910	1.40E-07	M252Y/N315D/G316Q/N434Y
F911	1.30E-07	M252Y/N315D/G316R/N434Y
F912	1.20E-07	M252Y/N315D/G316S/N434Y
F913	1.10E-07	M252Y/N315D/G316T/N434Y
F914	1.50E-07	M252Y/N315D/G316V/N434Y
F915	2.30E-07	M252Y/N315D/G316W/N434Y
F917	2.50E-07	M252Y/N286S/N434Y
F918	2.80E-07	M252Y/D280E/N384A/N389A/N434Y
F919	3.30E-07	M252Y/D280G/N384A/N389A/N434Y
F920	2.50E-07	M252Y/N286S/N384A/N389A/N434Y
F921	1.20E-07	M252Y/N286E/N384A/N389A/N434Y
F922	5.90E-08	L235K/S239K/M252Y/N286E/N434Y/Y436I
F923	6.00E-08	L235R/S239K/M252Y/N286E/N434Y/Y436I

Table 28-19 is the continuation of Table 28-18.

[Table 28-19]

F924	3.40E-08	L235K/S239K/M252Y/T307Q/Q311A/N434Y/Y436I
F925	3.20E-08	L235R/S239K/M252Y/T307Q/Q311A/N434Y/Y436I
F926	1.10E-07	L235K/S239K/M252Y/S254T/N434Y/Y436I
F927	1.00E-07	L235R/S239K/M252Y/S254T/N434Y/Y436I
F928	2.90E-08	M252Y/T307Q/Q311A/N434Y/Y436I
F929	2.90E-08	M252Y/S254T/T307Q/Q311A/N434Y/Y436I
F930	1.40E-07	P238D/T250V/M252Y/N286E/N434Y
F931	1.20E-07	P238D/T250V/M252Y/N434Y/Y436I
F932	3.20E-07	T250V/M252Y/N434Y
F933	3.00E-07	L234R/P238D/T250V/M252Y/N434Y
F934	3.10E-07	G236K/P238D/T250V/M252Y/N434Y
F935	3.20E-07	G237K/P238D/T250V/M252Y/N434Y
F936	3.20E-07	G237R/P238D/T250V/M252Y/N434Y
F937	3.10E-07	P238D/S239K/T250V/M252Y/N434Y
F938	1.60E-07	L235K/S239K/M252Y/N434Y/Y436V
F939	1.50E-07	L235R/S239K/M252Y/N434Y/Y436V
F940	1.50E-07	P238D/T250V/M252Y/N434Y/Y436V
F941	1.20E-08	M252Y/N286E/T307Q/Q311A/N434Y/Y436V
F942	4.20E-08	L235K/S239K/M252Y/T307Q/Q311A/N434Y/Y436V
F943	4.00E-08	L235R/S239K/M252Y/T307Q/Q311A/N434Y/Y436V
F944	1.70E-07	T250V/M252Y/N434Y/Y436V
F945	1.70E-08	T250V/M252Y/V308P/N434Y/Y436V
F946	4.30E-08	T250V/M252Y/T307Q/Q311A/N434Y/Y436V
F947	1.10E-08	T250V/M252Y/T307Q/V308P/Q311A/N434Y/Y436V
F954	5.30E-07	M252Y/N434Y/H435K/Y436V
F957	7.70E-07	M252Y/N434Y/H435N/Y436V
F960	8.00E-07	M252Y/N434Y/H435R/Y436V
F966	3.10E-07	M252Y/S254A/N434Y
F970	2.50E-06	M252Y/S254G/N434Y
F971	2.60E-06	M252Y/S254H/N434Y
F972	2.60E-07	M252Y/S254I/N434Y
F978	1.30E-06	M252Y/S254Q/N434Y
F980	1.80E-07	M252Y/S254V/N434Y
F987	4.00E-08	P238D/T250V/M252Y/T307Q/Q311A/N434Y/Y436V
F988	6.90E-08	P238D/T250V/M252Y/N286E/N434Y/Y436V
F989	1.40E-08	L235R/S239K/M252Y/V308P/N434Y/Y436V
F990	9.40E-09	L235R/S239K/M252Y/T307Q/V308P/Q311A/N434Y/Y436V
F991	1.30E-08	L235R/S239K/M252Y/N286E/T307Q/Q311A/N434Y/Y436V
F992	5.10E-08	L235R/S239K/M252Y/T307Q/Q311A/M428I/N434Y/Y436V
F993	3.80E-08	M252Y/T307Q/Q311A/N434Y/Y436V
F994	2.80E-07	M252Y/N325G/N434Y
F995	2.90E-07	L235R/P238D/S239K/M252Y/N434Y

Table 28-20 is the continuation of Table 28-19.

[Table 28-20]

F996	1.30E-07	L235R/P238D/S239K/M252Y/N434Y/Y436V
F997	3.80E-07	K248I/T250V/M252Y/N434Y/Y436V
F998	8.50E-07	K248Y/T250V/M252Y/N434Y/Y436V
F999	2.10E-07	T250V/M252Y/E258H/N434Y/Y436V
F1005		N325G
F1008	1.70E-07	L235R/S239K/T250V/M252Y/N434Y/Y436V
F1009	1.20E-08	L235R/S239K/T250V/M252Y/T307Q/V308P/Q311A/N434Y/Y436V
F1010	1.90E-07	L235R/S239K/M252Y/T307A/Q311H/N434Y
F1011	4.50E-08	T250V/M252Y/V308P/N434Y
F1012	4.70E-08	L235R/S239K/T250V/M252Y/V308P/N434Y
F1013	3.00E-08	T250V/M252Y/T307Q/V308P/Q311A/N434Y
F1014	3.20E-08	L235R/S239K/T250V/M252Y/T307Q/V308P/Q311A/N434Y
F1015	2.20E-08	L235R/S239K/M252Y/T307Q/V308P/Q311A/N434Y
F1016	3.80E-09	T250V/M252Y/N286E/T307Q/V308P/Q311A/N434Y/Y436V
F1017	4.20E-09	L235R/S239K/T250V/M252Y/N286E/T307Q/V308P/Q311A/N434Y/Y436V
F1018	3.20E-09	L235R/S239K/M252Y/N286E/T307Q/V308P/Q311A/N434Y/Y436V
F1019	3.40E-07	P238D/T250V/M252Y/N325G/N434Y
F1020	8.50E-08	P238D/T250V/M252Y/T307Q/Q311A/N325G/N434Y
F1021	3.30E-07	P238D/T250V/M252Y/N325A/N434Y
F1022		K326D/L328Y
F1023	4.40E-08	S239D/T250V/M252Y/T307Q/Q311A/N434Y/Y436V
F1024	4.00E-08	T250V/M252Y/T307Q/Q311A/K326D/L328Y/N434Y/Y436V
F1025	3.60E-08	S239D/T250V/M252Y/T307Q/Q311A/K326D/L328Y/N434Y/Y436V
F1026	8.40E-08	M252Y/T307A/Q311H/N434Y/Y436V
F1027	8.60E-08	L235R/S239K/M252Y/T307A/Q311H/N434Y/Y436V
F1028	4.60E-08	G236A/S239D/T250V/M252Y/T307Q/Q311A/N434Y/Y436V
F1029	5.10E-08	T250V/M252Y/T307Q/Q311A/I332E/N434Y/Y436V
F1030		I332E
F1031	5.30E-08	G236A/S239D/T250V/M252Y/T307Q/Q311A/I332E/N434Y/Y436V
F1032	4.30E-08	P238D/T250V/M252Y/T307Q/Q311A/N325G/N434Y/Y436V
F1033	1.00E-06	P238D/N434W
F1034	1.50E-08	L235K/S239K/M252Y/V308P/N434Y/Y436V
F1035	1.00E-08	L235K/S239K/M252Y/T307Q/V308P/Q311A/N434Y/Y436V
F1036	1.40E-08	L235K/S239K/M252Y/N286E/T307Q/Q311A/N434Y/Y436V
F1037	6.10E-08	L235K/S239K/M252Y/T307Q/Q311A/M428I/N434Y/Y436V
F1038	2.80E-07	L235K/P238D/S239K/M252Y/N434Y
F1039	1.30E-07	L235K/P238D/S239K/M252Y/N434Y/Y436V

Table 28-21 is the continuation of Table 28-20.

[Table 28-21]

F1040	2.00E-07	L235K/S239K/T250V/M252Y/N434Y/Y436V
F1041	1.40E-08	L235K/S239K/T250V/M252Y/T307Q/V308P/Q311A/N434Y/Y436V
F1042	2.00E-07	L235K/S239K/M252Y/T307A/Q311H/N434Y
F1043	5.20E-08	L235K/S239K/T250V/M252Y/V308P/N434Y
F1044	3.50E-08	L235K/S239K/T250V/M252Y/T307Q/V308P/Q311A/N434Y
F1045	2.50E-08	L235K/S239K/M252Y/T307Q/V308P/Q311A/N434Y
F1046	4.50E-09	L235K/S239K/T250V/M252Y/N286E/T307Q/V308P/Q311A/N434Y/Y436V
F1047	3.40E-09	L235K/S239K/M252Y/N286E/T307Q/V308P/Q311A/N434Y/Y436V
F1048	9.90E-08	L235K/S239K/M252Y/T307A/Q311H/N434Y/Y436V
F1050	3.50E-09	T250V/M252Y/N286E/T307Q/V308P/Q311A/M428I/N434Y/Y436V
F1051	3.90E-09	L235R/S239K/T250V/M252Y/N286E/T307Q/V308P/Q311A/M428I/N434Y/Y436V
F1052	3.20E-09	L235R/S239K/M252Y/N286E/T307Q/V308P/Q311A/M428I/N434Y/Y436V

[Reference Example 13] *In vivo* study of various Fc variant antibodies by steady-state infusion model using human FcRn transgenic mouse lineage 32

Fc variants generated in Reference Example 12 was tested for their ability to eliminate antigen from plasma in steady-state infusion model using human FcRn transgenic mouse lineage 32. Steady-state infusion model *in vivo* study was performed as described in Example 1, but human FcRn transgenic mouse lineage 32 was used instead of lineage 276, and monoclonal anti-mouse CD4 antibody was administered twice (before infusion pump was implanted and 14 days after antibody administration) or three times (before infusion pump was implanted and 10 and 20 days after antibody administration).

From the Fc variants described in Tables 28-1 to 28-21, selected antibody Fc variants listed below were expressed and purified by methods known to those skilled in the art as described in Reference Example 3:

Fv4-IgG1 comprising VH3-IgG1 and VL3-CK;

Fv4-IgG1-F11 comprising VH3-IgG1-F11 and VL3-CK;

Fv4-IgG1-F14 comprising VH3-IgG1-F14 and VL3-CK;

Fv4-IgG1-F39 comprising VH3-IgG1-F39 and VL3-CK;

Fv4-IgG1-F48 comprising VH3-IgG1-F48 and VL3-CK;

Fv4-IgG1-F140 comprising VH3-IgG1-F140 and VL3-CK;
Fv4-IgG1-F157 comprising VH3-IgG1-F157 and VL3-CK;
Fv4-IgG1-F194 comprising VH3-IgG1-F194 and VL3-CK;
Fv4-IgG1-F196 comprising VH3-IgG1-F196 and VL3-CK;
Fv4-IgG1-F198 comprising VH3-IgG1-F198 and VL3-CK;
Fv4-IgG1-F262 comprising VH3-IgG1-F262 and VL3-CK;
Fv4-IgG1-F264 comprising VH3-IgG1-F264 and VL3-CK;
Fv4-IgG1-F393 comprising VH3-IgG1-F393 and VL3-CK;
Fv4-IgG1-F424 comprising VH3-IgG1-F434 and VL3-CK; and
Fv4-IgG1-F447 comprising VH3-IgG1-F447 and VL3-CK.

These antibodies were administered to the human FcRn transgenic mouse lineage 32 at a dose of 1 mg/kg.

Fig. 49 describes the time course of plasma hsIL-6R concentration in the mouse. Compared to Fv4-IgG1, all the Fc variants having increased binding affinity to human FcRn at pH 7.0 exhibited reduction of plasma hsIL-6R concentration, therefore enhanced antigen elimination from plasma. Although the extent and durability of antigen concentration reduction was different among the Fc variants, all the variant consistently reduced the plasma hsIL-6R concentration as compared to IgG1 demonstrating that increased binding affinity to human FcRn at pH 7.0 would universally enhance the antigen elimination from plasma. Fig. 50 describes the time course of plasma antibody concentration in the mouse. Antibody pharmacokinetics was different among the Fc variants.

As described in Reference Example 9, amount of antigen eliminated from plasma per antibody is the important factor to evaluate the efficiency of antigen elimination by administrating the antibody Fc variants having increased binding affinity to human FcRn at pH 7.0. Therefore, time courses of value C (molar antigen/antibody ratio) for each antibody were described in Fig. 51. Fig. 52 describes the relationship between the binding affinity of Fc variants to human FcRn at pH 7.0 and value C (molar antigen/antibody ratio) at day 1 after administration of antibodies. This demonstrates that all the antibody Fc variants tested in this study have lower value C as compared to Fv4-IgG1. Since all the Fc variants tested in this study have binding affinity to human FcRn at pH 7.0 stronger than KD 3.0 μM, they achieved higher antigen elimination efficiency as compared to natural human IgG1. This was consistent with the results obtained in Reference Example 9 (Fig. 42).

Fig. 53 describes that among the Fc variants tested in this study, antibodies having Fc variant of F11, F39, F48, and F264 exhibited similar pharmacokinetics to IgG1. Since this study is conducted using human FcRn transgenic mouse, these Fc variants is expected to have long half-life similar to IgG1 also in human. Fig 54 describes the time course of plasma hsIL-6R

concentration in mice administered with antibodies having similar pharmacokinetics to natural human IgG1 (F11, F39, F48, and F264). These variants reduced the plasma hsIL-6R concentration as compared to IgG1 approximately 10-fold. Moreover, these antibodies reduced the hsIL-6R concentration below the baseline hsIL-6R concentration (concentration without antibody). Therefore, these antibodies would enable long-term elimination of antigen from plasma, and therefore long dosing intervals which would be preferable for antibody therapeutics for chronic disease.

Fig. 55 and 56 described the time course of plasma antibody concentration and plasma hsIL-6R concentration for IgG1, and Fc variant F157, F196 and F262, respectively. Surprisingly, although antibody pharmacokinetics of F157 and F262 showed significantly faster clearance from plasma as compared to natural human IgG1, F157 and F262 exhibited significant elimination of hsIL-6R from plasma. Specifically, plasma hsIL-6R concentration of F157 was below detection limit (1.56 ng/mL), from days 1 to 28 (except at day 14), and that of F262 was below detection limit (1.56 ng/mL) from days 14 to 28. On the other hand, for F196 with slower clearance of antibody compared to F157, antigen concentration started to increase at day 14 and returned back to baseline at day 28. Among the Fc variants tested in this study, F157 and F262 were the only Fc variants that were capable of reducing plasma hsIL-6R concentration below 1.56 ng/mL at day 28.

Such durable long-term effect of F157 and F262 is unexpected from the pharmacokinetics of the antibody, since antibodies were eliminated from plasma very rapidly as compared to natural human IgG1. In particular, plasma antibody concentration of F157 was not detected at day 21. Nevertheless, plasma hsIL-6R concentration continued to be reduced to a level lower than the detection limit of 1.56 ng/mL at days 21 and 28. The present invention is not limited to a particular theory, but this unexpected effect is considered to be due to the presence of the antibody at the surface of vascular endothelium cell as FcRn bound form. Although these antibodies showed low concentration in plasma, these antibodies is still present in the vascular compartment as FcRn bound form (which cannot be measured as a plasma antibody concentration). These FcRn bound antibody can still bind to the antigen in the plasma, and after FcRn mediated uptake of antigen/antibody complex, antigen is released within the endosome and degraded by the lysosome while the antibody is recycled back to the cell surface as FcRn bound form. Thus these FcRn bound antibody contribute to the antigen elimination. This explains the reason why these antibodies maintains antigen elimination capability even after the antibody concentration becomes low in plasma.

Industrial applicability

The present invention provides methods for promoting antigen uptake into cells by

using antigen-binding molecules, methods for increasing the number of times of antigen binding by one antigen-binding molecule, methods for promoting the reduction of plasma antigen concentration by administering antigen-binding molecules, and methods for improving plasma retention of antigen-binding molecules. By promoting antigen uptake into cells by an antigen-binding molecule, it becomes possible to not only promote the reduction of plasma antigen by administration of the antigen-binding molecule, but also improve the plasma retention of the antigen-binding molecule and increase the number of times of antigen binding by each of the antigen-binding molecule. Such antigen-binding molecules can exhibit more beneficial effects *in vivo* than typical antigen-binding molecules.

CLAIMS

1. An antibody comprising an antigen-binding domain and a human FcRn-binding domain, whose antigen-binding activity is different between two different calcium concentration conditions and is lower under a low calcium concentration condition than under a high calcium concentration condition, wherein the low calcium concentration is an ionized calcium concentration of 0.1 to 30 μM and the high calcium concentration is an ionized calcium concentration of 100 μM to 10 mM, and wherein a light chain or heavy chain of the antigen-binding domain comprises a calcium-binding motif derived from a human antibody, wherein in the calcium-binding motif in the light chain any four or more of the amino acids at positions 30, 31, 32, 50, and 92 according to Kabat's numbering have a metal-chelating activity.

2. The antibody of claim 1, wherein the calcium-binding motif is comprised at positions 30, 31, and/or 32 according to Kabat's numbering in the light chain.

3. The antibody of claim 1 or 2, wherein the calcium-binding motif is comprised at position 50 according to Kabat's numbering in the light chain.

4. The antibody of any one of claims 1 to 3, wherein the calcium-binding motif is comprised at position 92 according to Kabat's numbering in the light chain.

5. The antibody of any one of claims 1 to 4, wherein the antigen bound by the antibody is either IgA or glypican 3.

6. The antibody of claim 1, wherein the calcium-binding motif is comprised in the heavy chain CDR1, CDR2, and/or CDR3 of the antigen-binding domain.

7. The antibody of claim 6, wherein the calcium-binding motif is comprised at positions 95, 96, 100a, and/or 101 according to Kabat's numbering in the heavy chain CDR3.

8. The antibody of claim 6 or 7, wherein the antigen bound by the antibody is either IL-6R or IL-6.

9. The antibody of any one of claims 1 to 8, which comprises an FcRn-binding domain that has FcRn-binding activity in the neutral pH range.

10. The antibody of claim 9, wherein the FcRn-binding domain is an Fc region.

11. The antibody of claim 9 or 10, wherein one or more amino acids at positions 248, 250, 252, 254, 255, 256, 257, 258, 265, 286, 289, 297, 303, 305, 307, 308, 309, 311, 312, 314, 315, 317, 332, 334, 360, 376, 380, 382, 384, 385, 386, 387, 389, 424, 428, 433, 434, and 436 (EU numbering) in the amino acid sequence of the Fc region are different from those of the natural Fc region.

12. The antibody of claim 11, which comprises any one or combination of:
Met at amino acid position 237;
Ile at amino acid position 248;
Ala, Phe, Ile, Met, Gln, Ser, Val, Trp, or Tyr at amino acid position 250;
Phe, Trp, or Tyr at amino acid position 252;
Thr at amino acid position 254;
Glu at amino acid position 255;
Asp, Glu, or Gln at amino acid position 256;
Ala, Gly, Ile, Leu, Met, Asn, Ser, Thr, or Val at amino acid position 257;
His at amino acid position 258;
Ala at amino acid position 265;
Ala or Glu at amino acid position 286;
His at amino acid position 289;
Ala at amino acid position 297;
Ala at amino acid position 303;
Ala at amino acid position 305;
Ala, Asp, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Val, Trp, or Tyr at amino acid position 307;
Ala, Phe, Ile, Leu, Met, Pro, Gln, or Thr at amino acid position 308;
Ala, Asp, Glu, Pro, or Arg at amino acid position 309;
Ala, His, or Ile at amino acid position 311;
Ala or His at amino acid position 312;
Lys or Arg at amino acid position 314;
Ala, Asp, or His at amino acid position 315;
Ala at amino acid position 317;
Val at amino acid position 332;
Leu at amino acid position 334;

His at amino acid position 360;
Ala at amino acid position 376;
Ala at amino acid position 380;
Ala at amino acid position 382;
Ala at amino acid position 384;
Asp or His at amino acid position 385;
Pro at amino acid position 386;
Glu at amino acid position 387;
Ala or Ser at amino acid position 389;
Ala at amino acid position 424;
Ala, Asp, Phe, Gly, His, Ile, Lys, Leu, Asn, Pro, Gln, Ser, Thr, Val, Trp, or Tyr at amino acid position 428;
Lys at amino acid position 433;
Ala, Phe, His, Ser, Trp, or Tyr at amino acid position 434; or
His, Ile, Leu, or Val at amino acid position 436;
according to EU numbering in the Fc region.

13. A method of producing an antibody having at least one function selected from:
(i) function of promoting uptake of an antigen into cells,
(ii) function of binding to an antigen two or more times,
(iii) function of promoting the reduction of plasma antigen concentration, and
(iv) function of excellence in plasma retention,
wherein the method comprises the steps of (a) to (e) below:
(a) determining the antigen-binding activity of an antibody under a low calcium concentration condition;
(b) determining the antigen-binding activity of the antibody under a high calcium concentration condition;
(c) selecting an antibody that has a lower antigen-binding activity under the low calcium concentration condition than under the high calcium concentration condition;
(d) obtaining a gene encoding the antibody selected in step (c); and
(e) producing the antibody using the gene obtained in step (d),
wherein the low calcium concentration is an ionized calcium concentration of 0.1 to 30 μM and the high calcium concentration is an ionized calcium concentration of 100 μM to 10 mM.

14. A method of producing an antibody having at least one function selected from:
(i) function of promoting uptake of an antigen into cells,
(ii) function of binding to an antigen two or more times,
(iii) function of promoting the reduction of plasma antigen concentration, and
(iv) function of excellence in plasma retention,
wherein the method comprises the steps of (a) to (e) below:
(a) contacting an antigen with an antibody or a library of antibodies under a high calcium concentration condition;
(b) placing an antibody that binds to the antigen in step (a) under a low calcium concentration condition;
(c) obtaining an antibody that dissociates in step (b);
(d) obtaining a gene encoding the antibody obtained in step (c); and
(e) producing the antibody using the gene obtained in step (d),
wherein the low calcium concentration is an ionized calcium concentration of 0.1 to 30 μM and the high calcium concentration is an ionized calcium concentration of 100 μM to 10 mM.

15. A method of producing an antibody having at least one function selected from:
(i) function of promoting uptake of an antigen into cells,
(ii) function of binding to an antigen two or more times,
(iii) function of promoting the reduction of plasma antigen concentration, and
(iv) function of excellence in plasma retention,
wherein the method comprises the steps of (a) to (f) below:
(a) contacting an antigen with an antibody or a library of antibodies under a low calcium concentration condition;
(b) selecting an antibody that does not bind to the antigen in step (a);
(c) allowing the antibody selected in step (b) to bind to the antigen under a high calcium concentration condition;
(d) obtaining an antibody that bound to the antigen in step (c);
(e) obtaining a gene encoding the antibody obtained in step (d); and
(f) producing the antibody using the gene obtained in step (e),
wherein the low calcium concentration is an ionized calcium concentration of 0.1 to 30 μM and the high calcium concentration is an ionized calcium concentration of 100 μM to 10 mM.

16. The production method of any one of claims 13 to 15, which additionally comprises

the step of conferring or increasing the human FcRn-binding activity under a neutral pH condition by modifying an amino acid in the antibody.

17. The production method of any one of claims 13 to 15, which additionally comprises the step of reducing the antigen-binding activity under an acidic pH condition to be lower than that under a neutral pH condition by modifying an amino acid in the antibody.

18. The production method of any one of claims 13 to 15, wherein the low calcium concentration is an intraendosomal concentration of ionized calcium.

19. The production method of any one of claims 13 to 15, wherein the high calcium concentration is a plasma concentration of ionized calcium.

20. The production method of claim 17, wherein the amino acid modification in the antibody is modification by substituting at least one amino acid with histidine, or inserting at least one histidine into the antibody.

21. The production method of any one of claims 13 to 20, wherein the antigen bound by the antibody is an antigen selected from the group consisting of IL-6R, IL-6, IgA, human glypican 3, and IgE.

22. A pharmaceutical composition comprising:
the antibody of any one of claims 1 to 12 or an antibody produced by the production method of any one of claims 13 to 21, and
a pharmaceutically acceptable carrier.

23. The pharmaceutical composition of claim 22 for use in promoting internalization of the antigen into cells.

24. The pharmaceutical composition of claim 22 for use in promoting reduction of the antigen concentration in plasma.