DESCRIPTION

ANTIGEN-BINDING MOLECULE CAPABLE OF BINDING TO TWO OR MORE ANTIGEN MOLECULES REPEATEDLY

Technical Field

The present invention relates to methods for improving the pharmacokinetics of antigen-binding molecules and methods for increasing the number of times of antigen-binding of antigen-binding molecules, as well as antigen-binding molecules having improved pharmacokinetics, antigen-binding molecules having increased number of times of antigen-binding, and methods for producing such molecules.

Background Art

Antibodies are drawing attention as pharmaceuticals as they are highly stable in plasma and have few adverse effects. At present, a number of IgG-type antibody pharmaceuticals are available on the market and many more antibody pharmaceuticals are currently under development (Non-Patent Documents 1 and 2). Meanwhile, various technologies applicable to second-generation antibody pharmaceuticals have been developed, 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 introduction of 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).

The antigen-neutralizing capacity of a single antibody molecule depends on its affinity. By increasing the affinity, an antigen can be neutralized by smaller amount of an antibody. Various methods can be used to enhance the antibody affinity. 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 is impossible to completely neutralize antigen with the smaller amount of antibody 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. With the improvement of antibody pharmacokinetics or affinity maturation technology alone described above, there is thus a limitation in the reduction of the required antibody dose.

Accordingly, in order to sustain antibody's antigen-neutralizing effect for a target period with smaller amount of the antibody than the amount of antigen, a single antibody must neutralize multiple antigens. Methods for neutralizing multiple antigens with a single antibody include antigen inactivation using catalytic antibodies, which are antibodies conferred with a catalytic function. When the antigen is a protein, it can be inactivated by hydrolyzing its peptide bonds. An antibody can repeatedly neutralize antigens by catalyzing such hydrolysis (Non-Patent Document 8). There are many previous reports published on catalytic antibodies and technologies for producing them. However, there have been no reports of catalytic antibodies having sufficient catalytic activity as a pharmaceutical agent. Specifically, in an antibody *in vivo* study for a certain antigen, there has been no publication of catalytic antibodies which can produce a comparable or stronger effect even at low doses or produce a more prolonged effect even at a same dose as compared to an ordinary non-catalytic neutralizing antibody.

As described above, there have been no reports of antibodies that can produce a more superior *in vivo* effect than ordinary neutralizing antibodies through a single antibody neutralizing multiple antigen molecules. Thus, from the viewpoint of dose reduction and prolongation of the durability, there is a need for new technologies that permit the production of novel antibody molecules having a stronger *in vivo* effect than ordinary neutralizing antibodies by individually neutralizing multiple antigen molecules.

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

[Prior art documents]

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

[Problems to be Solved by the Invention]

The above noted circumstances led to the discoveries of the present invention. Accordingly, an objective of the present invention is to provide methods for binding antigen-binding molecules to the antigens multiple times and methods for improving the pharmacokinetics of antigen-binding molecules, as well as antigen-binding molecules that are capable of binding to the antigens multiple times, antigen-binding molecules having improved pharmacokinetics, pharmaceutical compositions containing such antigen-binding molecules, and methods for producing such molecules and compositions.

[Means for Solving the Problems]

Dedicated studies on methods for binding polypeptides having antigen-binding ability, such as antigen-binding molecules, to the antigens multiple times, and methods for improving

the half-lives of such molecules in plasma (blood) (improving their pharmacokinetics) were conducted herein. As a result, it was discovered that if the antigen-binding activity of an antigen-binding molecule at the early endosomal pH is lower than its antigen-binding activiy at the pH of plasma (blood), it would be able to bind to antigens multiple times and have a longer half-life in plasma.

Accordingly, the present invention relates to methods for binding antigen-binding molecules to antigens multiple times, methods for improving the pharmacokinetics of antigen-binding molecules, and methods for producing antigen-binding molecules with improved pharmacokinetics; the present invention also relates to antigen-binding molecules that are capable of binding to antigens multiple times and antigen-binding molecules with improved pharmacokinetics. More specifically, the present invention provides:

[1] an antigen-binding molecule having a KD(pH5.8)/KD(pH7.4) value, defined as the ratio of KD for the antigen at pH 5.8 and KD for the antigen at pH 7.4, of 2 or higher;

[2] the antigen-binding molecule of [1], wherein the KD(pH5.8)/KD(pH7.4) value is 10 or higher;

[3] the antigen-binding molecule of [1], wherein the KD(pH5.8)/KD(pH7.4) value is 40 or higher;

[4] the antigen-binding molecule of any one of [1] to [3], wherein at least one amino acid of the antigen-binding molecule has been substituted with histidine, or at least one histidine has been inserted into the antigen-binding molecule;

[5] the antigen-binding molecule of any one of [1] to [4], wherein the antigen-binding molecule has an antagonistic activity;

[6]. the antigen-binding molecule of any one of [1] to [5], wherein the antigen-binding molecule binds to a membrane antigen or a soluble antigen;

[7] the antigen-binding molecule of any one of [1] to [6], wherein the antigen-binding molecule is an antibody;

[8] a pharmaceutical composition comprising the antigen-binding molecule of any one of [1] to [7];

[9] a method for improving the pharmacokinetics of an antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4;

[10] a method for increasing the number of times of antigen-binding for an antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4;

[11] a method for increasing the number of antigens that can be bound by an antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4;

[12] a method for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4;

[13] a method for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4;

[14] a method for increasing the ability of an antigen-binding molecule to eliminate an antigen in plasma by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4;

[15] the method of any one of [9] to [14], wherein the KD(pH5.8)/KD(pH7.4) value, defined as the ratio of KD for the antigen at pH 5.8 and KD for the antigen at pH 7.4, is 2 or higher;

[16] the method of any one of [9] to [14], wherein the KD(pH5.8)/KD(pH7.4) value is 10 or higher;

[17] the method of any one of [9] to [14], wherein the KD(pH5.8)/KD(pH7.4) value is 40 or higher;

[18] a method for improving the pharmacokinetics of an antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[19] a method for increasing the number of times of antigen-binding for an antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[20] a method for increasing the number of antigens that can be bound by an antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[21] a method for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[22] a method for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[23] a method for increasing the ability of an antigen-binding molecule to eliminate an antigen in plasma by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;

[24] the method of any one of [18] to [23], wherein the histidine substitution or insertion increases the KD(pH5.8)/KD(pH7.4) value, defined as the ratio of the antigen-binding activity at pH 5.8 and the antigen-binding activity at pH 7.4, as compared to the KD(pH5.8)/KD(pH7.4) value before the histidine substitution or insertion;

[25] the method of any one of [9] to [24], wherein the antigen-binding molecule has an antagonistic activity;

[26] the method of any one of [9] to [25], wherein the antigen-binding molecule binds to a membrane antigen or a soluble antigen;

[27] the method of any one of [9] to [26], wherein the antigen-binding molecule is an antibody;

[28] a method of screening for an antigen-binding molecule, which comprises the steps of:

(a) determining the antigen-binding activity of an antigen-binding molecule at pH 6.7 to pH 10.0;

(b) determining the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5; and

(c) selecting an antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than the antigen-binding activity at pH 4.0 to pH 6.5;

[29] the screening method of [28], which comprises the step of selecting an antibody whose antigen-binding activity at pH 6.7 to pH 10.0 is twice or higher that of the antigen-binding activity at pH 4.0 to pH 6.5;

[30] a method of screening for an antigen-binding molecule, which comprises the steps of:

(a) binding an antigen-binding molecule to an antigen under a condition of pH 6.7 to pH 10.0;

(b) placing the antigen-binding molecule that bound to the antigen of (a) under a condition of pH 4.0 to pH 6.5; and

(c) obtaining an antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5;

[31] a method of screening for an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:

(a) binding an antigen-binding molecule to an antigen-immobilized column under the condition of a first pH;

(b) eluting the antigen-binding molecule that had bound to the column at the first pH from the column under the condition of a second pH; and

(c) collecting the eluted antigen-binding molecule;

[32] a method of screening for an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:
(a) binding an antigen-binding molecule library to an antigen-immobilized column under the condition of a first pH;
(b) eluting the antigen-binding molecule from the column under the condition of a second pH;
(c) amplifying the gene encoding the eluted antigen-binding molecule; and
(d) obtaining the eluted antigen-binding molecule.
[33] the screening method of [31] or [32], wherein the first pH is 6.7 to 10.0 and the second pH is 4.0 to 6.5;
[34] the screening method of any one of [28] to [33], wherein at least one or more amino acids of the antigen-binding molecule has been substituted with histidine, or at least one histidine has been inserted into the antigen-binding molecule;
[35] the screening method of any one of [28] to [33], for obtaining an antigen-binding molecule that is superior in retention in the plasma;
[36] the screening method of any one of [28] to [33], for obtaining an antigen-binding molecule that is capable of binding to an antigen two or more times;
[37] the screening method of any one of [28] to [33], for obtaining an antigen-binding molecule that is capable of binding to more antigens as compared to the number of its antigen-binding sites;
[38] the screening method of any one of [28] to [33], for obtaining an antigen-binding molecule that dissociates an extracellularly-bound antigen within a cell.
[39] the screening method of any one of [28] to [33], for obtaining an antigen-binding molecule that is bound to an antigen and internalized into a cell, and released to the outside of the cell in an antigen-free form;
[40] the screening method of any one of [28] to [33], for obtaining an antigen-binding molecule that has increased ability to eliminate an antigen in plasma;
[41] the screening method of any one of [28] to [40], wherein the antigen-binding molecule is used as a pharmaceutical composition;
[42] the screening method of any one of [28] to [41], wherein the antigen-binding molecule is an antibody;
[43] a method for producing an antigen-binding molecule, which comprises the steps of:
(a) determining the antigen-binding activity of an antigen-binding molecule at pH 6.7 to pH 10.0;
(b) determining the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5;
(c) selecting the antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5;

(d) obtaining the gene encoding the antigen-binding molecule selected in (c); and
(e) producing the antigen-binding molecule using the gene obtained in (d);
[44] a method for producing an antigen-binding molecule, which comprises the steps of:
(a) binding an antigen-binding molecule to an antigen under a condition of pH 6.7 to pH 10.0;
(b) allowing the antigen-binding molecule bound to the antigen of (a) to stand under a condition of pH 4.0 to pH 6.5;
(c) collecting the antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5;
(d) obtaining the gene encoding the antigen-binding molecule obtained in (c); and
(e) producing the antigen-binding molecule using the gene obtained in (d);
[45] a method for producing an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:
(a) binding an antigen-binding molecule to an antigen-immobilized column under the first pH condition;
(b) eluting the antigen-binding molecule, which is bound to the column at the first pH, from the column under a second pH condition;
(c) collecting the eluted antigen-binding molecule;
(d) obtaining the gene encoding the antigen-binding molecule obtained in (c); and
(e) producing the antigen-binding molecule using the gene obtained in (d);
[46] a method for producing an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:
(a) binding an antigen-binding molecule library to an antigen-immobilized column under the first pH condition;
(b) eluting the antigen-binding molecule from the column under the second pH condition;
(c) amplifying the gene encoding the eluted antigen-binding molecule;
(d) collecting the eluted antigen-binding molecule;
(e) obtaining the gene encoding the antigen-binding molecule collected in (d); and
(f) producing the antigen-binding molecule using the gene obtained in (e);
[47] the production method of [45] or [46], wherein the first pH is 6.7 to 10.0 and the second pH is 4.0 to 6.5.
[48] the production method of any one of [43] to [47], which further comprises the step of substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule;
[49] the production method of any one of [43] to [48], wherein the antigen-binding molecule is an antibody;

[50] a pharmaceutical composition comprising an antigen-binding molecule produced by the production method of any one of [43] to [49].

[Effects of the Invention]

The present invention provides methods for making single antigen-binding molecules to repeatedly bind to multiple antigen molecules. When an antigen-binding molecule binds to multiple antigen molecules, the pharmacokinetics of the antigen-binding molecule can be improved and such molecule can exert more superior *in vivo* effects than those of ordinary antigen-binding molecules.

Brief Description of the Drawings

FIG. 1 is a diagram depicting a degradation pathway of antibodies bound to membrane-bound antigen.

FIG. 2 is a diagram depicting a mechanism by which IgG molecules are salvaged by FcRn.

FIG. 3 is a schematic diagram depicting the re-binding of IgG molecules to new antigen following dissociation from membrane-bound antigen within endosomes.

FIG. 4 is a schematic diagram depicting the re-binding of IgG molecules to new antigen following dissociation from soluble antigen within endosomes.

FIG. 5 is a diagram depicting the process of panning using an antigen-immobilized column.

FIG. 6 presents graphs depicting the results of phage ELISA for clones acquired by column panning. The upper graph depicts WT and the lower graph depicts CL5.

FIG. 7 is a graph depicting the biological neutralization activity of pH-dependently-binding anti-IL-6 receptor antibodies.

FIG. 8 presents graphs depicting results of Biacore sensorgram for binding of pH-dependently-binding anti-IL-6 receptor antibodies to soluble IL-6 receptor at pH 7.4. The top graph depicts WT; the second graph from the top depicts H3pI/L73; the third graph from the top depicts H170/L82; and the bottom depicts CLH5/L73.

FIG. 9 presents graphs depicting results of Biacore sensorgram for binding of pH-dependently-binding anti-IL-6 receptor antibodies to soluble IL-6 receptor at pH 5.8. The top graph depicts WT; the second graph from the top depicts H3pI/L73; the third graph from the top depicts H170/L82; and the bottom graph depicts CLH5/L73.

FIG. 10 presents graphs depicting results of Biacore sensorgram for association (pH 7.4) and dissociation (pH 5.8) of pH-dependently-binding anti-IL-6 receptor antibodies to membrane-type IL-6 receptor. The top graph depicts WT; the second graph from the top

depicts H3pI/L73; the third graph from the top depicts H170/L82; and the bottom graph depicts CLH5/L73.

FIG. 11 is a Biacore sensorgram indicating repeated binding of pH-dependently-binding anti-IL-6 receptor antibodies to SR344.

FIG. 12 is a graph depicting the total amount of bound antigen in a repetitive binding experiment of pH-dependently-binding anti-IL-6 receptor antibodies to SR344.

FIG. 13 is a graph depicting time courses of antibody plasma concentrations of pH-dependently-binding anti-IL-6 receptor antibodies in human IL-6 receptor transgenic mice.

FIG. 14 is a graph depicting time courses of antibody plasma concentrations of pH-dependently-binding anti-IL-6 receptor antibodies in cynomolgus monkeys.

FIG. 15 is a graph depicting time courses of CRP concentrations in cynomolgus monkeys, in relation to pH-dependently-binding anti-IL-6 receptor antibodies.

FIG. 16 is a graph depicting time courses of unbound-type cynomolgus monkey IL-6 receptor concentrations in cynomolgus monkeys, in relation to pH-dependently-binding anti-IL-6 receptor antibodies.

FIG. 17 presents graphs depicting results of Biacore sensorgram of association (pH 7.4) and dissociation (pH 5.8) of pH-dependently-binding anti-IL-6 receptor antibodies to membrane-type IL-6 receptor. In order moving from the top, the results for WT, H3pI/L73-IgG1, Fv2-IgG1, and Fv4-IgG1 are shown.

FIG. 18 is a graph depicting time courses of plasma antibody concentrations of pH-dependently-binding anti-IL-6 receptor antibodies (WT, H3pI/L73-IgG1, Fv2-IgG1, and Fv4-IgG1) in human IL-6 receptor transgenic mice.

FIG. 19 presents graphs depicting results of Biacore sensorgram for association (pH 7.4) and dissociation (pH 5.8) of pH-dependently-binding anti-IL-6 receptor antibodies to membrane-type IL-6 receptor. Moving from the top down, the results for WT, Fv4-IgG1, Fv4-IgG2, and Fv4-M58 are shown.

FIG. 20 is a graph showing time courses of antibody plasma concentrations of pH-dependently-binding anti-IL-6 receptor antibodies (WT, Fv4-IgG1, Fv4-IgG2, and Fv4-M58) in human IL-6 receptor transgenic mice..

FIG. 21 presents graphs depicting results of Biacore sensorgram of association (pH 7.4) and dissociation (pH 5.8) of pH-dependently-binding anti-IL-6 receptor antibodies to membrane-type IL-6 receptor. Moving from the top down, the results for Fv1-M71, Fv1-M73, Fv3-M71, and Fv3-M73 are shown.

FIG. 22 is a graph depicting time courses of antibody plasma concentrations of pH-dependently-binding anti-IL-6 receptor antibodies in cynomolgus monkeys, during

administration of H3pI/L73-IgG1, Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M73, and Fv4-M73 at 0.5 mg/kg and during administration of high affinity Ab at 1.0 mg/kg.

FIG. 23 is a graph depicting time courses of CRP concentrations in cynomolgus monkeys, in relation to pH-dependently-binding anti-IL-6 receptor antibodies (H3pI/L73-IgG1-, Fv1-M71-, Fv1-M73-, Fv2-IgG1-, Fv3-M73-, Fv4-M73-, and high-affinity-Ab-administered groups).

FIG. 24 is a graph depicting time courses of unbound-type cynomolgus monkey IL-6 receptor concentrations in cynomolgus monkeys, in relation to pH-dependently-binding anti-IL-6 receptor antibodies (H3pI/L73-IgG1-, Fv1-M71-, Fv1-M73-, Fv2-IgG1-, Fv3-M73-, Fv4-M73-, and high-affinity-Ab-administered groups).

FIG. 25 is a diagram depicting FR1, FR2, FR3, and FR4 along with CDR1, CDR2, and CDR3 of heavy chains (VH1, VH2, VH3, VH4) and light chains (VL1, VL2, VL3). Asterisks indicate locations where amino acid mutations exist in the aligned sequences.

FIG. 26 presents a Biacore sensorgram depicting the pH-dependent binding of an anti-IL-6 antibody, Anti-IL6 clone 2, to IL-6 at pH 7.4 and pH 5.5. The curves in the sensorgram at pH 7.4 correspond to 100, 50, 25, 12.5, and 6.25 ng/mL IL-6, from above.

FIG. 27 presents a Biacore sensorgram depicting the pH-dependent binding of an anti-IL-31 receptor antibody, Anti-IL31R clone 1, to the IL-31 receptor at pH 7.4 and pH 5.5. The curves in the sensorgram at pH 5.5 correspond to 100, 50, 25, and 12.5 ng/mL IL-31 receptor, from above.

FIG. 28 depicts the time course of plasma antibody concentration after intravenous administration of a mixture solution containing SR344 and an anti-human IL-6 receptor antibody to mouse.

FIG. 29 depicts the time course of plasma SR344 concentration after intravenous administration of a mixture solution containing SR344 and an anti-human IL-6 receptor antibody to mouse.

Mode for Carrying Out the Invention

The present invention provides methods for increasing the number of times of antigen-binding in antigen-binding molecules. More specifically, the present invention provides methods for increasing the number of times of antigen-binding in antigen-binding molecules by impairing the antigen-binding ability of the antigen-binding molecules at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for increasing the number of times of antigen-binding in antigen-binding molecules by substituting histidine for at least one amino acid in the antigen-binding molecules or inserting at least one histidine into the antigen-binding molecules. In addition, the present invention provides

methods for increasing the number of times of antigen-binding in antigen-binding molecules by substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecules.

The present invention also provides methods for increasing the number of antigens that can be bound by an antigen-binding molecule. More specifically, the present invention provides methods for increasing the number of antigens that can be bound by an antigen-binding molecule by impairing the antigen-binding ability at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for increasing the number of antigens that can be bound by an antigen-binding molecule by substituting histidine for at least one amino acid in the antigen-binding molecules or inserting at least one histidine into the antigen-binding molecules. In addition, the present invention provides methods for increasing the number of antigens that can be bound by an antigen-binding molecule through substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecules.

The present invention also provides methods for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule. More specifically, the present invention provides methods for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by impairing the antigen-binding ability at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by substituting histidine for at least one amino acid in the antigen-binding molecule or inserting at least one histidine into the antigen-binding molecule. In addition, the present invention provides methods for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule through substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecule.

The present invention also provides methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell. More specifically, the present invention provides methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, by impairing the antigen-binding ability at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, by substituting histidine for at least one amino acid in the antigen-binding molecule or inserting at least one histidine into the antigen-binding molecule. In addition, the present invention provides methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an

antigen-free form to the outside of the cell, through substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecule.

The present invention also provides methods for increasing the ability of an antigen-binding molecule to eliminate antigens in plasma. More specifically, the present invention provides methods for increasing the ability of an antigen-binding molecule to eliminate antigens in plasma by impairing the antigen-binding ability at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for increasing the ability of an antigen-binding molecule to eliminate antigens in plasma by substituting histidine for at least one amino acid in the antigen-binding molecules or inserting at least one histidine into the antigen-binding molecules. In addition, the present invention provides methods for increasing the ability of an antigen-binding molecule to eliminate antigens in plasma through substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecule.

The present invention also provides methods for improving the pharmacokinetics of antigen-binding molecules. More specifically, the present invention provides methods for improving the pharmacokinetics of antigen-binding molecules (prolonging the retention in plasma) by impairing the antigen-binding ability at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for improving the pharmacokinetics of antigen-binding molecules by substituting histidine for at least one amino acid in the antigen-binding molecules or inserting at least one histidine into the antigen-binding molecules. In addition, the present invention provides methods for improving the pharmacokinetics of antigen-binding molecules by substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecules.

Further, the present invention provides methods for increasing the ability of the antigen-binding molecules to eliminate antigens in plasma. More specifically, the present invention provides methods for increasing the ability of the antigen-binding molecules to eliminate antigens in plasma by impairing the antigen-binding ability of the antigen-binding molecules at acidic pH as compared to that at neutral pH. Furthermore, the present invention provides methods for increasing the ability of the antigen-binding molecules to eliminate antigens in plasma by substituting at least one amino acid in the antigen-binding molecules with histidine or inserting at least one histidine into the antigen-binding molecules. In addition, the present invention provides methods for increasing the ability of the antigen-binding molecules to eliminate antigens in plasma by substituting, deleting, adding, and/or inserting amino acids in the antibody constant region of antigen-binding molecules.

Herein, "improvement of the pharmacokinetics", "amelioration of the pharmacokinetics", "superior pharmacokinetics" are interchangeable with "improvement of the

retention in plasma (blood)", "amelioration of the retention in plasma (blood)", and "superior retention in plasma (blood)", respectively, and these phrases are synonymous.

Herein, impairing the antigen-binding activity at acidic pH as compared to that at neutral pH means that the antigen-binding ability of an antigen-binding molecule at pH 4.0 to pH 6.5 is impaired as compared to that at pH 6.7 to pH 10.0, preferably that the antigen-binding activity of an antigen-binding molecule at pH 5.5 to pH 6.5 is impaired as compared to that at pH 7.0 to pH 8.0, and more preferably that the antigen-binding activity of an antigen-binding molecule at pH 5.8 is impaired as compared to that at pH 7.4. Accordingly, in the present invention, acidic pH is typically pH 4.0 to pH 6.5, preferably pH 5.5 to pH 6.5, and more preferably pH 5.8. Alternatively, in the present invention, neutral pH is typically pH 6.7 to pH 10.0, preferably pH 7.0 to pH 8.0, and more preferably pH 7.4.

Herein, the phrase "impairing the antigen-binding ability of an antigen-binding molecule at acidic pH as compared to that at neutral pH" is interchangable with the phrase "increasing the antigen-binding ability of an antigen-binding molecule at neutral pH as compared to that at acidic pH". In other words, in the present invention, the difference in the antigen-binding ability of an antigen-binding molecule should be increased between acidic and neutral pHs. For example, the value of KD(pH5.8)/KD(pH7.4) should be increased, as described below. The difference in the antigen-binding ability of an antigen-binding molecule between acidic and neutral pHs may be increased, for example, by either or both, impairing the antigen-binding ability at acidic pH and increasing the antigen-binding ability at neutral pH.

Conditions other than the pH for determining the antigen-binding activity can be selected appropriately by those skilled in the art, and the conditions are not particularly limited. The antigen-binding activity can be determined, for example, under conditions of MES buffer and 37°C as described in the Examples herein. Furthermore, the antigen-binding activity of an antigen-binding molecule can be determined by methods known to those skilled in the art, for example, using Biacore (GE Healthcare) or the like, as described in the Examples herein. When the antigen is a soluble antigen, the activity of binding to the soluble antigen can be assessed by injecting the antigen as an analyte onto a chip immobilized with the antigen-binding molecule. Alternatively, when the antigen is a membrane antigen, the activity of binding to the membrane antigen can be assessed by injecting the antigen-binding molecule as an analyte onto an antigen-immobilized chip.

In the present invention, the difference in the antigen-binding activity between acidic and neutral pHs is not particularly limited as long as the antigen-binding activity at acidic pH is lower than that at neutral pH. However, the value of KD(pH5.8)/KD(pH7.4), which is a ratio of dissociation constant (KD) against an antigen at pH 5.8 and that at pH 7.4, is preferably 2 or greater, more preferably 10 or greater, and still more preferably 40 or greater. The upper limit

of KD(pH5.8)/KD(pH7.4) value is not particularly limited, and may be any value, for example, 400, 1,000, or 10,000, as long as the molecule can be produced by technologies of those skilled in the art. When the antigen is a soluble antigen, the antigen-binding activity can be presented in terms of the dissociation constant (KD). Alternatively, when the antigen is a membrane antigen, the antigen-binding activity can be presented in terms of the apparent dissociation constant. 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, or FACS.

Alternatively, it is possible to use, for example, k_d, a dissociation rate constant, as an indicator for the difference in the antigen-binding activity between acidic and neutral pHs. When the dissociation rate constant (k_d) is used as an indicator for the difference in the binding activity instead of the dissociation constant (KD), the value of k_d(pH5.8)/k_d(pH7.4), which is a ratio of dissociation rate constant (k_d) against an antigen at pH 5.8 and that at 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 k_d(pH5.8)/k_d(pH7.4) value is not particularly limited, and may be any value, for example, 50, 100, or 200, as long as the molecule can be produced by technologies common to those skilled in the art.

When the antigen is a soluble antigen, the antigen-binding activity can be presented in terms of the dissociation rate constant (k_d). Alternatively, when the antigen is a membrane antigen, the antigen-binding activity can be presented in terms of the 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) or FACS.

In the present invention, when the antigen-binding activity of an antigen-binding molecule is determined at different pHs, it is preferred that the measurement conditions except for pH are constant.

The methods for impairing the antigen-binding activity of an antigen-binding molecule at pH 5.8 as compared to that at pH 7.4 (methods for conferring the pH-dependent binding ability) are not particularly limited and may be any methods. Such methods include, for example, methods for impairing the antigen-binding activity at pH 5.8 as compared to that at pH 7.4 by substituting histidine for amino acids in the antigen-binding molecule or inserting histidine into the antigen-binding molecule. It is already known that an antibody can be conferred with a pH-dependent antigen-binding activity by substituting histidine for amino acids in the antibody (FEBS Letter, 309(1), 8588 (1992)). Such histidine mutation (substitution) or insertion sites are not particularly limited, and any site is acceptable as long as the antigen-binding activity at pH 5.8 is lowered than that at pH 7.4 (the value of

KD(pH5.8)/KD(pH7.4) gets greater) as compared to before mutation or insertion. When the antigen-binding molecule is an antibody, such sites include, for example, sites within an antibody variable region. The appropriate number of histidine mutation or insertion sites can be appropriately determined by those skilled in the art. Histidine may be substituted or inserted at a single site, or two or more sites. It is also possible to introduce non-histidine mutation (mutation with amino acids other than 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, antigen-binding molecules whose KD(pH5.8)/KD(pH7.4) is increased as compared to before mutation can be selected from an antigen-binding molecule library with random histidine mutation or insertion.

When histidine is substituted for amino acids of an antigen-binding molecule or inserted between amino acids of the molecule, it is preferred, but not required, 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. 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" means that even after histidine substitution or insertion, the antigen-binding molecule 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 of before histidine substitution or insertion. When the antigen-binding activity of the antigen-binding molecule has been impaired due to histidine substitution or insertion, the antigen-binding activity may be adjusted by introducing substitution, deletion, addition, and/or insertion of one or more amino acids into the antigen-binding molecule so that the antigen-binding activity becomes comparable to that before histidine substitution or insertion. The present invention also includes such antigen-binding molecules having a comparable binding activity as a result of substitution, deletion, addition, and/or insertion of one or more amino acids after histidine substitution or insertion.

Alternative methods for impairing the antigen-binding activity of an antigen-binding molecule at pH 5.8 as compared to that at pH 7.4 include methods of substituting non-natural amino acids for amino acids in an antigen-binding molecule or inserting non-natural amino acids into amino acids of an antigen-binding molecule. It is known that the 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 described above. Such non-natural amino acid substitution and/or insertion may be introduced simultaneously with the histidine

substitution and/or insertion described above. Any non-natural amino acids may be used in the present invention. It is possible to use non-natural amino acids known to those skilled in the art.

Furthermore, when the antigen-binding molecule is a substance having an antibody constant region, alternative methods for impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4 include methods for modifying the antibody constant region contained in the antigen-binding molecule. Such methods for modifying the antibody constant region include, for example, methods for substituting a constant region described in the Examples herein.

Alternative methods for modifying the antibody constant region include, for example, methods to assess various constant region isotypes (IgG1, IgG2, IgG3, and IgG4) and select an isotype that impairs the antigen-binding activity at pH 5.8 (increases the dissociation rate at pH 5.8). Alternatively, methods include those for impairing the antigen-binding activity at pH 5.8 (increasing the dissociation rate at pH 5.8) by substituting amino acids in the amino acid sequence of a wild-type isotype (the amino acid sequence of wild type IgG1, IgG2, IgG3, or IgG4). The sequence of the hinge region of an 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 impair the antigen-binding activity at pH 5.8 (to increase the dissociation rate at pH 5.8) by considering the type of antigen or epitope. Furthermore, since the difference in the hinge region amino acid sequence has a significant influence on the antigen-binding activity, preferred amino acid substitution sites in the amino acid sequence of a wild type isotype are assumed to be within the hinge region.

When the antigen-binding activity of the antigen-binding substance at pH 5.8 is weakened compared to that at pH 7.4 (when KD(pH5.8)/KD(pH7.4) value is increased) by the above described methods and such, it is generally preferable that the KD(pH5.8)/KD(pH7.4) value be two times or more, more preferably five times or more, and even more preferably ten times or more as compared to that of the original antibody, although the invention is not particularly limited thereto.

Herein, the "improvement of the pharmacokinetics" means prolongation of the time required for the elimination of the antigen-binding molecule from plasma (for example, reaching the state where the antigen-binding molecule cannot return to the plasma due to degradation in cells, or other reasons) after administration to an animal such as human, mouse, rat, monkey, rabbit, or dog, as well as prolongation of the plasma retention time of the antigen-binding molecule being in a form capable of binding to antigens (for example, being in an antigen-free form) during the period until it is eliminated from the plasma after administration. Even if an

antigen-binding molecule is circulated in plasma, it cannot bind to an antigen when it already binds to another antigen. Accordingly, the period where the antigen-binding molecule can newly binds to another antigen is prolonged (the chance to bind another antigen increases) by prolonging the period where the antigen-binding molecule is in an antigen-free form. This makes it possible to shorten the period where the antigen is free from antigen-binding molecules *in vivo* (in other words, to prolong the period where the antigen is bound by an antigen-binding molecule). For example, the ratio of antigens bound to antigen-binding molecules against the antigens in the body in plasma (total of antigen molecules bound to and free from the antigen-binding molecules) generally decreases in a certain period of time after the administration of the antigen-binding molecules. However, such decrease can be suppressed (for example, the degree of decrease can be made smaller) by prolonging the retention time of the antigen-binding molecules in a form capable of binding to antigens. This results in an increase in the ratio of antigens bound to antigen-binding molecules against the antigens in the body in a certain period of time after antibody administration.

Specifically, in the present invention, the "improvement of the pharmacokinetics" does not necessarily mean the prolongation (extension) of the time required for the elimination of the antigen-binding molecule after administration. Even if the time required for the elimination of the antigen-binding molecule after administration remains unchanged, the pharmacokinetics can be said "improved" in the present invention if

the plasma retention time of the antigen-binding molecule being in a form capable of binding to an antigen (for example, the antigen-binding molecule being in an antigen-free form) is prolonged;

the period where the antigen is free from an antigen-binding molecule in the body is shortened (in other words, the period where the antigen-binding molecule is bound to an antigen is prolonged); and

the ratio of antigens bound to antigen-binding molecules against the antigens in the body is increased. Thus, in the present invention, the "improvement of the pharmacokinetics" encompasses at least:

(1) prolongation of the time required for the elimination of the antigen-binding molecule from plasma after administration of the antigen-binding molecule;

(2) prolongation of the plasma retention time of the antigen-binding molecule in a form capable of binding to an antigen after administration of the antigen-binding molecule;

(3) shortening of the period where the antigen is free from an antigen-binding molecule in the body after administration of the antigen-binding molecule (prolongation of the period where the antigen-binding molecule is bound to an antigen in the body); and

(4) increase in the ratio of antigens bound to antigen-binding molecules to the antigen in the body.

When the antigen is a soluble antigen present in plasma, even if the pharmacokinetics of the antigen-binding molecule (rate of elimination from plasma) is equivalent, there are cases where elimination of antigen bound to the antigen-binding molecule is accelerated. Reducing the pharmacokinetics of the antigen (accelerating elimination from plasma) results in the relative improvement of the pharmacokinetics of the antigen-binding molecule, and thus, leads to the prolongation of the time of the antigen-binding molecule present in plasma in a form capable of binding to antigens. Thus, in one embodiment, the "improvement of the pharmacokinetics" of antigen-binding molecules of the present invention includes increasing the rate of eliminating soluble antigens from plasma after administration of the antigen-binding molecules (the ability of the antigen-binding molecule to eliminate antigens from plasma).

In the present invention, when the antigen is a membrane antigen, whether a single antigen-binding molecule binds to multiple antigens can be assessed by testing whether the pharmacokinetics of the antigen-binding molecule is improved. Whether the "pharmacokinetics is improved" can be assessed by the following method. For example, whether the time required for the elimination of an antigen-binding molecule after administration is prolonged can be assessed by determining any one of parameters for the antigen-binding molecule, such as half-life in plasma, mean plasma retention time, and clearance in plasma ("Pharmacokinetics: Enshu-niyoru Rikai (Understanding through practice)" Nanzando). For example, when the half-life in plasma or mean plasma retention time of an antigen-binding molecule administered to mice, rats, monkeys, rabbits, dogs, humans, or other animals is prolonged, the pharmacokinetics of the antigen-binding molecule is judged to be improved. These parameters can be determined by methods known to those skilled in the art. For example, the parameters can be appropriately assessed by noncompartmental analysis using pharmacokinetics analysis software WinNonlin (Pharsight) according to the appended instruction manual.

Alternatively, whether the plasma retention time of an antigen-binding molecule in a form capable of binding to antigens after administration of the antigen-binding molecule is prolonged can be assessed by measuring the plasma concentration of the antigen-free antigen-binding molecule and determining any one of parameters for the antigen-free antigen-binding molecule, such as half-life in plasma, mean plasma retention time, and clearance in plasma. The concentration of the antigen-free antigen-binding molecule in plasma can be measured by methods known to those skilled in the art. For example, such measurements are described in Clin Pharmacol. 2008 Apr;48(4):406-17.

Furthermore, whether the period where an antigen is free from the antigen-binding molecules in the body after administration of the antigen-binding molecules is shortened (the period where the antigen-binding molecule is bound to an antigen in the body is prolonged) can be assessed by determining the plasma concentration of the unbound antigen that is free from antigen-binding molecules, and considering the period where the concentration of free antigen in plasm or the amount ratio of free antigen against the total antigen remains low. The plasma concentration of the free antigen or amount ratio of free antigen against total antigen can be determined by methods known to those skilled in the art. For example, such measurements are described in Pharm Res. 2006 Jan;23(1):95-103. Alternatively, when the antigen exerts some function *in vivo*, whether the antigen is bound by an antigen-binding molecule that neutralizes the antigen's function (antagonistic molecule) can be assessed by testing whether the function of the antigen is neutralized. Whether the function of the antigen is neutralized can be assessed by assaying an *in vivo* marker that reflects the function of the antigen. Whether the antigen is bound by an antigen-binding molecule that activates the function of the antigen (agonistic molecule) can be assessed by assaying an *in vivo* marker that reflects the function of the antigen.

There is no particular limitation on the determination of the plasma concentration of free antigen and amount ratio of free antigen against total antigen, and *in vivo* marker assay, but the determination is preferably carried out after a certain period following administration of an antigen-binding substance. In the present invention, such a period following administration of an antigen-binding substance is not particularly limited, and an appropriate period can be determined by those skilled in the art depending on the properties of the administered antigen-binding substance and the like. Examples of the period are: one day after administration of an antigen-binding substance; three days after administration of an antigen-binding substance, seven days after administration of an antigen-binding substance, 14 days after administration of an antigen-binding substance, and 28 days after administration of an antigen-binding substance.

In the present invention, it is preferred to improve the pharmacokinetics in human. Even when the plasma retention in human is difficult to determine, it can be predicted based on the plasma retention in mice (for example, normal mice, human antigen-expressing transgenic mice, and human FcRn-expressing transgenic mice) or monkeys (for example, cynomolgus monkeys).

Methods for determining the retention in plasma are not particularly limited. The determination can be carried out, for example, according to the methods described in the Examples herein.

Whether an antigen-binding molecule is capable of binding to antigens multiple times can be assessed by testing whether the antigen bound to an antigen-binding molecule under the

same neutral condition as plasma dissociates under the same acidic condition as endosome and how many antigens the antigen-binding molecule can rebind to under the neutral condition. Specifically, the assessment can be carried out by allowing the antigen-binding molecule and antigen to form a complex under the neutral condition, exposing the complex to an acidic condition for a predetermined period, and then testing whether the antigen-binding molecule can rebind to an antigen under the neutral condition, using a device for assaying antigen-binding molecule-antigen reactions, such as Biacore. When the antigen-binding capacity of the antigen-binding molecule conferred with the pH-dependent binding ability has been improved to twice that of the antigen-binding molecule before modification, the number of times of binding of the antigen-binding molecule conferred with the pH-dependent binding ability can be judged to be increased to twice that of the antigen-binding molecule before modification. Alternatively, when the antigen is a membrane antigen and thus the antigen-binding molecule is eliminated from plasma through antigen-mediated uptake and degradation in a lysosome, whether the number of times of binding of the antigen-binding molecule conferred with the pH-dependent binding ability is increased as compared to that before modification can be assessed by comparing the pharmacokinetics or duration of antigen binding between the antigen-binding molecule conferred with the pH-dependent binding ability and the antigen-binding molecule before modification. For example, when the antigen-binding duration of the antigen-binding molecule conferred with the pH-dependent binding ability is prolonged twice that of the antigen-binding molecule before modification, the number of times of binding of the antigen-binding molecule conferred with the pH-dependent binding ability is judged to be increased to twice that of the antigen-binding molecule before modification. Alternatively, when the plasma concentration of an unbound antigen, which is free from the antigen-binding molecule, is determined and the period where the plasma concentration of the free antigen or the amount ratio of the free antigen against the total antigen remains low is prolonged to twice, the number of times of binding of the antigen-binding molecule conferred with the pH-dependent binding ability is judged to be increased to twice that of the antigen-binding molecule before modification.

When the antigen is a soluble antigen, if the antigen bound to an antigen-binding molecule under the neutral condition in plasma dissociates in an endosome, and the antigen-binding molecule returns to the plasma, the antigen-binding molecule can again bind to an antigen under the neutral condition in plasma. Thus, an antigen-binding molecule that has the characteristics to dissociate with an antigen in acidic condition of an endosome is capable of binding to antigens multiple times. Compared to when the antigen bound to an antigen-binding molecule does not dissociate in an endosome (the antigen remains bound to the antigen-binding molecule when returning to plasma), when the antigen bound to an antigen-binding molecule

dissociates in endosomes, the antigen is delivered to a lysosome and then degraded, and thus, the rate of elimination of the antigen from plasma increases. That is, it is also possible to determine whether the antigen-binding molecule is capable of binding to antigens multiple times using the rate of elimination of antigen from plasma as an index. The rate of elimination of the antigen from plasma can be determined, for example, by administering the antigens (e.g., membrane antigen) and antigen-binding molecules *in vivo*, and then measuring the concentration of antigens in plasma. When an antigen (e.g., membrane antigen) is produced or secreted *in vivo*, the plasma antigen concentration is reduced if the rate of elimination of the antigen from plasma is increased. Thus, it is also possible to determine whether the antigen-binding molecule is capable of binding to antigens multiple times using the plasma antigen concentration as an index.

Herein, "increasing the number of times of antigen-binding of the antigen-binding molecule" means that the number of cycles is increased when taking as one cycle the process where an antigen-binding molecule administered to human, mouse, monkey, or such binds to an antigen and is internalized into a cell. Specifically, herein, "the antigen-binding molecule binds twice to an antigen" means that the antigen-binding molecule bound by an antigen is internalized into a cell and released in an antigen-free form to the outside of the cell, and the released antigen-binding molecule rebinds to another antigen and is internalized into a cell again.

When the antigen-binding molecule is internalized into a cell, it may be in a form bound by a single antigen, or two or more antigens.

Herein, "the number of times of antigen-binding of an antigen-binding molecule is increased" does not necessarily mean that the number of times of antigen-binding increases in every antigen-binding molecules. For example, among antigen-binding molecules in an antigen-binding-molecule composition, the proportion of antigen-binding molecules that bind to antigens twice or more times may increase, or the average number of binding events of antigen-binding molecules in an antigen-binding-molecule composition may increase.

In the present invention, it is preferred that the number of times of antigen-binding of an antigen-binding molecule increases when the molecule is administered to a human. However, when it is difficult to determine the number of times of antigen-binding in human, the number in human may be predicted based on the results obtained by *in vitro* assay or measurement using mice (for example, antigen-expressing transgenic mice and human FcRn-expressing transgenic mice) or monkeys (for example, cynomolgus monkeys).

In the present invention, it is preferred that an antigen-binding molecule binds to antigens twice or more times. For example, it is preferred that, of the antigen-binding molecules in an antigen-binding-molecule composition, at least 10% or more, preferably 30% or more, more preferably 50% or more, and still more preferably 80% or more (for example, 90% or more, 95% or more, and so on) bind to antigens twice or more times.

Herein, "increasing the number of antigens that can be bound by an antigen-binding molecule" means increasing the number of antigens that can be bound by an antigen-binding molecule during the period until the antigen-binding molecule is degraded in a lysosome of a cell after administration of the antigen-binding molecule to an animal such as human, mouse, or monkey.

In general, antibodies such as IgG have two binding domains, and thus a single antibody binds to a maximum of two antigens. An antibody bound to antigen(s) is internalized into a cell, and the antibody and antigen(s) are degraded in a lysosome. In general, antibodies such as IgG can bind to a maximum of two antigens. When the antigen-binding activity of an antigen-binding molecule such as an antibody at the endosomal pH is impaired as compared to that at the plasma pH by the methods of the present invention, the antigen-binding molecule such as an antibody internalized into a cell dissociates the antigen and is released to the outside of the cell, and thus can bind to another antigen again. In other words, the methods of the present invention enable for an antigen-binding molecule to bind to more antigens than the number of its antigen-binding sites. Specifically, by using the methods of the present invention, for example, IgG having two antigen-binding sites can bind to three or more antigens, preferably four or more antigens, during a period until the antibody is degraded after administration. For example, when the antibody is a neutralizing antibody, "increasing the number of antigens that can be bound by an antigen-binding molecule" is interchangable with "increasing the number of antigens that the antigen-binding molecule can neutralize". Thus, "bind" can be replaced with "neutralize" when the antibody is a neutralizing antibody.

In the present invention, "increasing the number of antigens that can be bound by an antigen-binding molecule" does not necessarily mean increasing the number of antigens that can be bound by every antigen-binding molecule. For example, the average number of antigens that can be bound by an antigen-binding molecule in an antigen-binding-molecule composition may increase, or the proportion of antigen-binding molecules that can bind to more antigens than the number of their antigen-binding sites may increase.

In the present invention, it is preferred that the number of antigens that can be bound by an antigen-binding molecule increases when the molecule is administered to a human. However, when it is difficult to determine the number in human, it may be predicted based on the results obtained by *in vitro* assay or measurement using mice (for example, antigen-expressing transgenic mice and human FcRn-expressing transgenic mice) or monkeys (for example, cynomolgus monkeys). When the antibody is a neutralizing antibody, the above-described number of times of antigen-binding of the antigen-binding molecule is generally assumed to correlates with the number of antigens which can be neutralized by an antigen-binding molecule. Thus, the number of antigens which can be neutralized by an

antigen-binding molecule can be determined by the same methods described above for determining the number of times of binding of an antigen-binding molecule.

Furthermore, the present invention provides methods for binding an antigen-binding molecule to antigens twice or more times in the body, by administering an antigen-binding molecule whose antigen-binding activity at acidic pH is lower than that at neutral pH.

The present invention also relates to methods for neutralizing antigens that are greater in number than the number of antigen-binding sites of an antigen-binding molecule having the neutralizing activity, by administering the antigen-binding molecule whose antigen-binding activity at acidic pH is lower than that at neutral pH. Preferably, the present invention relates to methods for neutralizing three or more antigens, preferably four or more antigens by administering IgG whose antigen-binding activity at acidic pH is lower than that at neutral pH.

The present invention also relates to methods for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by impairing the antigen-binding ability of the antigen-binding molecule at acidic pH as compared to that at neutral pH. In the present invention, the antigen may be dissociated from the antigen-binding molecule anywhere within a cell; however, it is preferred that the antigen is dissociated within an early endosome. In the present invention, "an antigen is dissociated within a cell from an extracellularly-bound antigen-binding molecule" does not necessarily mean that every antigen internalized into a cell via binding to the antigen-binding molecule is dissociated from the antigen-binding molecule within the cell. It is acceptable that the proportion of antigen that is dissociated from the antigen-binding molecule within a cell increases when compared to before impairing the antigen-binding ability of the antigen-binding molecule at acidic pH as compared to that at neutral pH.

Furthermore, the present invention relates to methods for enhancing the intracellular binding of an antigen-binding molecule free from an antigen to FcRn by impairing the antigen-binding ability of the antigen-binding molecule at acidic pH as compared to that at neutral pH. In general, FcRn binds to an antigen-binding molecule within an endosome. However, an antigen-binding molecule bound to a membrane antigen is assumed not to bind to FcRn. Thus, in a preferred embodiment, when the antigen is a membrane-bound antigen, the present invention includes methods for enhancing the endosomal dissociation of antigens from antigen-binding molecules and thus enhancing the FcRn binding of the antigen-binding molecules, by impairing the antigen-binding ability of an antigen-binding molecule at the endosomal pH (acidic pH) as compared to that at the plasma pH (neutral pH). When the antigen is a soluble antigen, the antigen-binding molecule can bind to FcRn in the presence or absence of the antigen. If dissociation of the antigen from the antigen-binding molecule within endosomes can be promoted by impairing the antigen-binding ability of the antigen-binding

molecule at intraendosomal (acidic) pH as compared to that at plasma (neutral) pH, the FcRn binding of the antigen-binding molecule that is "free from an antigen" can be enhanced by the methods of the present invention.

Regardless of whether an antigen is membrane-bound or soluble, if an antigen-binding molecule free from an antigen can return to plasma with FcRn, the antigen-binding molecule can bind to the antigen again. By repeating this process, the antigen-binding molecule can bind to the antigen multiple times. In the present invention, "enhancing the FcRn binding of an antigen-binding molecule within a cell" does not necessarily mean that every antigen-binding molecule binds to FcRn. It is acceptable that the proportion of an antigen-binding molecule free from an antigen that binds to FcRn within a cell increases when compared to before impairing the antigen-binding ability of the antigen-binding molecule at the endosomal pH as compared to that at the plasma pH. Preferred antigen-binding molecules in the methods of the present invention for enhancing the intracellular binding between the antigen-binding molecule and FcRn include, for example, antigen-binding molecules that bind to membrane-bound antigens (membrane antigens) such as membrane proteins. Other preferable antigen-binding molecules include antigen-binding molecules that bind to soluble antigens such as soluble proteins.

The methods of enhancing the binding of an antigen-binding molecule and FcRn within a cell are alternatively expressed as the methods of promoting the FcRn binding of an antigen-binding molecule within a cell, for example, within endosomes.

Furthermore, the present invention relates to methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, by impairing the antigen-binding ability of the antigen-binding molecule at acidic pH as compared that at neutral pH. In the present invention, "releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell" does not necessarily mean that every antigen-binding molecule, which has been bound to an antigen and internalized into a cell, is released in an antigen-free form to the outside of the cell. It is acceptable that the proportion of antigen-binding molecules that are released to the outside of the cell increases when compared to before impairing the antigen-binding ability of the antigen-binding molecule at acidic pH as compared to that at neutral pH. It is preferred that the antigen-binding molecule released to the outside of a cell retains the antigen-binding ability. Furthermore, the method of releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell can also be referred to as a method of conferring to the antigen-binding molecule a property that the antigen-binding molecule becomes more easily

released to the outside of the cell in an antigen-free form when the antigen-binding molecule is bound to an antigen and internalized into a cell.

Furthermore, the present invention relates to methods for increasing the ability of the antigen-binding molecules to eliminate antigens in plasma by impairing the antigen-binding ability of the antigen-binding molecules at acidic pH as compared to that at neutral pH. In the present invention "the ability to eliminate antigens in plasma" refers to the ability to eliminate from plasma antigens that are present in plasma, when the antigen-binding molecules are administered *in vivo* or are secreted *in vivo*. Thus, in the present invention, "increasing the ability of the antigen-binding molecule to eliminate antigen in plasma" means that the rate of elimination of antigens from plasma when the antigen-binding molecules are administered *in vivo* is accelerated as compared to that before lowering the antigen-binding ability of the antigen-binding molecules at acidic pH as compared to that at neutral pH. Whether the ability of the antigen-binding molecule to eliminate antigens in plasma is increased can be determined by, for example, administering soluble antigens and antigen-binding molecules *in vivo*, and then measuring the concentration of soluble antigens in plasma. When the concentration of soluble antigens in plasma after the administration of soluble antigens and antigen-binding molecules is reduced by lowering the antigen-binding ability of the antigen-binding molecule at acidic pH than that at neutral pH, it can be determined that the ability of the antigen-binding molecule to eliminate antigens in plasma is increased.

The present invention also relates to methods for improving the pharmacokinetics of an antigen-binding molecule by substituting histidine or non-natural amino acid for at least one amino acid in the antigen-binding molecule or inserting histidine or non-natural amino acid into the molecule.

Furthermore, the present invention provides methods for increasing the number of times of antigen-binding of an antigen-binding molecule by substituting histidine or non-natural amino acid for at least one amino acid in the antigen-binding molecule or inserting histidine or non-natural amino acid into the molecule.

In addition, the present invention relates to methods for increasing the number of antigens that can be bound by an antigen-binding molecule by substituting histidine or non-natural amino acid for at least one amino acid in the antigen-binding molecule or inserting histidine or non-natural amino acid into the molecule.

The present invention also provides methods for dissociating an antigen within a cell from an extracellularly-bound antigen-binding molecule by substituting at least one amino acid in the antigen-binding molecule with histidine or non-natural amino acid, or inserting histidine or non-natural amino acid into the molecule.

The present invention also provides methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell by substituting at least one amino acid in the antigen-binding molecule with histidine or non-natural amino acid, or inserting histidine or non-natural amino acid into the molecule.

The present invention also provides methods for increasing the ability of the antigen-binding molecule to eliminate antigens in plasma by substituting at least one amino acid in the antigen-binding molecule with histidine or non-natural amino acid, or inserting histidine or non-natural amino acid into the molecule.

The site of histidine or non-natural amino acid mutation (substitution, insertion, etc.) is not particularly limited. A histidine or non-natural amino acid may be substituted or inserted at any site. Preferred sites of histidine or non-natural amino acid substitution or insertion include, for example, sites within a region that has an impact on the antigen-binding ability of the antigen-binding molecule. For example, when the antigen-binding molecule is an antibody, such sites include an antibody variable region or CDR. The number of histidine or non-natural amino acid mutations is not particularly limited. Histidine or non-natural amino acid may be substituted or inserted at a single site, or at two or more sites. Furthermore, a deletion, addition, insertion, and/or substitution of other amino acids may be introduced simultaneously with the histidine or non-natural amino acid substitution or insertion.

In the present invention, when the antigen-binding molecule is an antibody, possible sites of histidine or non-natural amino acid substitution include, for example, sites within the CDR sequence or sequence responsible for the CDR structure of an antibody. Such sites include, for example, the sites listed below. The amino acid positions are numbered based on the Kabat numbering (Kabat EA *et al.*, (1991) Sequences of Proteins of Immunological Interest, NIH).

Heavy chain: H27, H31, H32, H33, H35, H50, H58, H59, H61, H62, H63, H64, H65, H99, H100b, and H102

Light chain: L24, L27, L28, L32, L53, L54, L56, L90, L92, and L94

Among the above sites, H32, H61, L53, L90, and L94 could be universal modification sites.

When the antigen is the IL-6 receptor (*e.g.*, human IL-6 receptor), preferable modification sites include the following. However, the modification sites are not particularly limited thereto.

Heavy chain: H27, H31, H32, H35, H50, H58, H61, H62, H63, H64, H65, H100b, and H102

Light chain: L24, L27, L28, L32, L53, L56, L90, L92, and L94

When histidine or non-natural amino acid is substituted at multiple sites, preferred combinations of substitution sites include, for example, the combination of H27, H31, and H35; combination of H27, H31, H32, H35, H58, H62, and H102; combination of L32 and L53; and combination of L28, L32, and L53. In addition, preferred combinations of substitution sites of heavy and light chains include the combination of H27, H31, L32, and L53.

When the antigen is IL-6 (*e.g.*, human IL-6), preferable modification sites include the following. However, the modification sites are not particularly limited thereto.

Heavy chain: H32, H59, H61, and H99

Light chain: L53, L54, L90, and L94

When the antigen is the IL-31 receptor (*e.g.*, human IL-31 receptor), preferable modification sites include H33. However, the modification sites are not particularly limited thereto.

Regarding the above sites, only one site may be substituted with histidine or non-natural amino acid. Alternatively, multiple sites may be substituted with histidine or non-natural amino acid.

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

The antigen-binding molecules of the present invention are not particularly limited as long as they have the specific binding activity to an antigen of interest. Preferred antigen-binding molecules of the present invention include, for example, substances having an antigen-binding domain of an antibody. The antigen-binding domain of an antibody includes, for example, CDR and variable region. When the antigen-binding domain of an antibody is CDR, the antigen-binding molecule may include all of the six CDRs of a whole antibody, or one, or two or more of them. Alternatively, when an antigen-binding molecule includes CDR as a binding domain of an antibody, the CDR may include amino acid deletion, substitution, addition, and/or insertion, or may be a partial CDR.

Furthermore, when the antigen-binding molecule includes an antibody constant region, the present invention relates to methods for improving the pharmacokinetics of antigen-binding molecules by modification (for example, amino acid substitution, deletion, addition, and/or insertion) of the antibody constant region in the antigen-binding molecule.

In addition, when the antigen-binding molecule includes an antibody constant region, the present invention provides methods for increasing the number of times of antigen-binding of an antigen-binding molecule by modification (for example, amino acid substitution, deletion, addition, and/or insertion) of the antibody constant region in the antigen-binding molecule.

Furthermore, when the antigen-binding molecule includes an antibody constant region, the present invention relates to methods for increasing the number of antigens that can be bound

by an antigen-binding molecule by modification (for example, amino acid substitution, deletion, addition, and/or insertion) of the antibody constant region in the antigen-binding molecule.

Furthermore, when the antigen-binding molecule includes an antibody constant region, the present invention relates to methods for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by modification (for example, amino acid substitution, deletion, addition, and/or insertion) of the antibody constant region in the antigen-binding molecule.

Furthermore, when the antigen-binding molecule includes an antibody constant region, the present invention relates to methods for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, by modification (for example, amino acid substitution, deletion, addition, and/or insertion) of the antibody constant region in the antigen-binding molecule.

Furthermore, when the antigen-binding molecule includes an antibody constant region, the present invention relates to methods for increasing the ability of an antigen-binding molecule to eliminate antigens in plasma by modification (for example, amino acid substitution, deletion, addition, and/or insertion) of the antibody constant region in the antigen-binding molecule.

In a preferred embodiment, the antigen-binding substance of the present invention includes antigen-binding substances including an FcRn-binding region. After internalized into cells, antigen-binding substances including an FcRn-binding region can return to the plasma by the FcRn salvage pathway. The FcRn-binding region is preferably a domain that directly binds to FcRn. Preferred FcRn-binding region includes, for example, antibody Fc regions. However, the FcRn-binding region of the present invention may be a region that can bind to a polypeptide having the ability to bind to FcRn such as albumin or IgG, since such region that can bind to the polypeptide having FcRn-binding ability can binds indirectly to FcRn via albumin, IgG, etc.

Antigens recognized by antigen-binding molecules such as antibodies of interest in the methods of the present invention are not particularly limited. Such antibodies of interest may recognize any antigen. Antibodies whose pharmacokinetics is to be improved by the methods of the present invention include, for example, antibodies that recognize membrane antigens such as receptor proteins (membrane-bound receptors and soluble receptors) and cell surface markers, and antibodies that recognize soluble antigens such as cytokines. Preferred examples of membrane antigens of the present invention include membrane proteins. Examples of soluble antigens of the present invention include soluble proteins. Antigens recognized by antibodies whose pharmacokinetics is to be improved by the methods of the present invention include, for example, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15, IL-31, IL-23, IL-2 receptor, IL-6 receptor, OSM receptor, gp130, IL-5 receptor, CD40, CD4, Fas,

osteopontin, CRTH2, CD26, PDGF-D, CD20, monocyte chemotactic factor, CD23, TNF-α, HMGB-1, α4 integrin, ICAM-1, CCR2, CD11a, CD3, IFNγ, BLyS, HLA-DR, TGF-β, CD52, and IL-31 receptor. Particularly preferred antigens include IL-6 receptor.

Furthermore, the antigen-binding molecule of interest in the methods of the present invention includes antigen-binding molecules having an antagonistic activity (antagonistic antigen-binding molecules) and antigen-binding molecules having an agonistic activity (agonistic antigen-binding molecules). In a preferred embodiment, the antigen-binding molecule includes antagonistic antigen-binding molecules, in particular, antagonistic antigen-binding molecules that recognize membrane antigens such as receptors, or soluble antigens such as cytokines. For example, an antagonistic antigen-binding molecule that recognizes a receptor inhibits the ligand-receptor binding by binding to the receptor, and thus inhibits the signaling mediated via the receptor.

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 includes both antigen-binding activity (antigen-binding region) and FcRn-binding region. In particular, preferred antigen-binding molecule of the present invention includes a region that binds to human FcRn. The antigen-binding molecule including both antigen-binding activity and FcRn-binding region includes, for example, antibodies. The antibodies preferred in the context of the present invention include, for example, IgG antibodies. When the antibody to be used is an IgG antibody, the type of IgG is not limited; the IgG belonging to any isotype (subclass) such as IgG1, IgG2, IgG3, or IgG4 can be used. Furthermore, amino acid mutations (*e.g.*, M73) may be introduced into the constant region of any of these IgG isotypes. Amino acid mutations to be introduced include, for example, those potentiate or impair the binding to Fcγ receptor (Proc Natl Acad Sci U S A. 2006 Mar 14;103(11):4005-10) and those potentiate or impair the binding to FcRn (J Biol Chem. 2001 Mar 2;276(9):6591-604), 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 of IgG2.

When the antigen-binding molecule of interest of 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 a modified antibody, for example, a chimeric antibody, and in particular, a modified antibody including amino acid substitution in the sequence of a humanized antibody, etc. The antibodies also include bispecific antibodies, antibody modification products linked with various molecules, and polypeptides including 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 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).

Bispecific antibody refers to an antibody that has, in the same antibody molecule, variable regions 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 including antibody fragments include, for example, Fab fragments, F(ab')2 fragments, scFv (Nat Biotechnol. 2005 Sep;23(9):1126-36), domain antibodies (dAb) (WO 2004/058821, WO 2003/002609), scFv-Fc (WO 2005/037989), dAb-Fc, and Fc fusion proteins. Of these, molecules including an Fc domain have the activity of binding to FcRn, and are therefore suitable for use in the methods discovered in the present invention.

Further, the antigen-binding molecules that are applicable to the present invention may be antibody-like molecules. An antibody-like 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). If these antibody-like molecules can bind to target molecules in a pH-dependent manner, it is possible for a single molecule to bind multiple target molecules.

Furthermore, the antigen-binding molecule may be a receptor protein or a receptor-Fc fusion protein that binds to a target, including, for example, TNFR-Fc fusion protein, IL1R-Fc fusion protein, VEGFR-Fc fusion protein, and CTLA4-Fc fusion protein (Nat Med. 2003 Jan;9(1):47-52; BioDrugs. 2006;20(3):151-60). If such receptor proteins and receptor-Fc fusion proteins can bind to target molecules in a pH-dependent manner, it is possible for a single molecule to bind multiple target molecules.

Moreover, the antigen-binding molecule may be an artificial ligand protein or artificial ligand fusion protein that binds to a target and has the neutralizing effect, and includes, for

example, mutant IL-6 (EMBO J. 1994 Dec 15;13(24):5863-70). If such artificial ligand proteins and artificial ligand fusion proteins can bind to target molecules in a pH-dependent manner, it is possible for a single molecule to bind multiple target molecules.

Furthermore, the antibodies of the present invention may include modified sugar chains. Antibodies with modified sugar chains include, for example, antibodies with modified glycosylation (WO 99/54342), antibodies that are deficient in fucose that is added to the sugar chain (WO 00/61739; WO 02/31140; WO 2006/067847; WO2 006/067913), and antibodies having sugar chains with bisecting GlcNAc (WO 02/79255).

Although the methods of the present invention are not limited to any specific theory, the relationship between making the antigen-binding ability at acidic pH weaker as compared to that at neutral pH, the improvement of the pharmacokinetics, and the multiple-time binding to the antigen can be explained as follows, for instance.

For example, when the antibody 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 and 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 typical 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 FcRn which is known as a salvage receptor of IgG molecules. When taken up into endosomes via pinocytosis, IgG molecules bind to FcRn expressed in the endosomes under the acidic condition in the endosomes. While IgG molecules that did not bind to FcRn transfer to lysosomes where they are degraded, IgG molecules that bound to FcRn translocate to the cell surface and return again in the plasma by dissociating from FcRn under the neutral condition in the plasma.

Alternatively, when the antigen is an antigen 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 cells via FcRn. However, since the antibodies are released to the outside of cells,

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 typical antibodies, one molecule of IgG antibody cannot bind to three or more antigen molecules.

The present inventors reasoned that, when antibodies that bound to antigens such as membrane antigens are taken up into endosomes by internalization, while the antibodies that are kept bound to the antigens translocate to lysosomes and are degraded, the IgG antibodies whose antigens dissociated in the endosomes could bind to FcRn that are expressed in the endosomes. Specifically, the present inventors discovered that an antibody that strongly binds to an antigen in the plasma but weakly binds to the antigen within the endosome can bind to an antigen in plasma and be taken up while kept forming a complex with the antigen into endosomes in the cells via internalization; dissociate from the antigen in the endosome; then bind to FcRn and translocate to the cell surface; and return again in the plasma in a state not bound to antigens to neutralize multiple membrane-bound antigens. Furthermore, the present inventors discovered that an antibody having the property of strongly binding to antigens in the plasma but weakly binding to antigens in the endosome can dissociate from the antigens in the endosome even when the antibody had bound to antigens such as soluble antigens; therefore, they are released again into the plasma in a state not bound to antigens and can neutralize multiple soluble antigens.

In particular, the present inventors noted that the pH in the plasma was different from the pH in the endosomes, and thus discovered that antibodies that strongly bind to antigens under plasma pH condition but that weakly bind to antigens under endosomal pH condition were superior in retention in the plasma, because one antibody molecule could bind to multiple antigens.

The endosomes, which are membrane vesicles, form networks in the cytoplasm of eukaryotic cells and are responsible for the metabolism of macromolecules in the process from the cell membrane to the lysosomes. The pH in the endosomes has been reported be generally an acidic pH of 5.5 to 6.0 (Nat Rev Mol Cell Biol. 2004 Feb;5(2):121-32). Meanwhile, the pH in the plasma is known to be almost neutral (normally, pH 7.4).

Accordingly, an antigen-binding molecule whose antigen-binding activity at acidic pH is weaker than the antigen-binding activity at neutral pH binds to the antigen in the plasma which have a neutral pH, is taken up into cells, and then dissociates from the antigen in the endosomes which have an acidic pH. The antigen-binding molecule that dissociated from the antigen binds to FcRn, translocates to the cell surface, and returns again in the plasma in a state not bound to antigens. As a result, the antigen-binding molecule can bind to antigens multiple times, and the pharmacokinetics is improved.

<Antigen-binding molecule substances>

Furthermore, the present invention provides antigen-binding molecules whose antigen-binding activity at pH 4.0 to pH 6.5 is lower than that at pH 6.7 to pH 10.0, preferably antigen-binding molecules whose antigen-binding activity at pH 5.0 to pH 6.0 is lower than that at pH 7.0 to 8.0. Specifically, antigen-binding molecules whose antigen-binding activity at pH 4.0 to pH 6.5 is lower than that at pH 6.7 to pH 10.0 include, for example, antigen-binding molecules whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4. Antigen-binding molecules whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4 can also be expressed as antigen-binding molecules whose antigen-binding activity at pH 7.4 is higher than that at pH 5.8.

As for the antigen-binding molecules of the present invention whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4, so long as the antigen-binding activity at pH 5.8 is lower than the binding at pH 7.4, there is no limitation on the difference in binding activity, and the antigen-binding activity at pH 5.8 only need to be lower, even slightly.

A preferred embodiment of an antigen-binding molecule of the present invention whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4 includes antigen-binding molecules whose antigen-binding activity at pH 7.4 is twice or greater than that at pH 5.8. A more preferred embodiment of the antigen-binding molecule includes antigen-binding molecules whose antigen-binding activity at pH 7.4 is ten times or greater than that at pH 5.8. A still more preferred embodiment of the antigen-binding molecule includes antigen-binding molecules whose antigen-binding activity at pH 7.4 is 40 times or greater than that at pH 5.8.

Specifically, in a preferred embodiment, the antigen-binding molecule of the present invention has antigen-binding activity at pH 5.8 that is lower than that at pH 7.4, wherein the value of KD(pH5.8)/KD(pH7.4), which is a ratio of KD for the antigen at pH 5.8 and that at 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(pH5.8)/KD(pH7.4) value is not particularly limited, and may be any value, for example, 400, 1000, or 10000, as long as production is possible using the technologies of those skilled in the art.

In another preferred embodiment, the antigen-binding molecule of the present invention whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4, has a value of k_d(pH5.8)/k_d(pH7.4), which is a ratio of the k_d for the antigen at pH 5.8 and the k_d for the antigen at pH 7.4, that is 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(pH5.8)/k_d(pH7.4) value is not particularly limited, and may be any value, for example, 50, 100, or 200, as long as production is possible using the technologies of those skilled in the art.

Conditions other than the pH at which the antigen-binding activity is measured can be appropriately selected by those skilled in the art, and the conditions are not particularly limited;

however, the measurements can be carried out, for example, under conditions of MES buffer and 37°C, as described in the Examples. Furthermore, the antigen-binding activity of an antigen-binding molecule can be determined by methods known to those skilled in the art, for example, using Biacore T100 (GE Healthcare) or the like, as described in the Examples.

It is presumed that such an antigen-binding molecule, which weakly binds to an antigen at acidic pH, easily dissociates from the antigen under the endosomal acidic condition, and that after internalization into cells, it binds to FcRn and is easily released to the outside of the cells. The antigen-binding molecule released to the outside of the cells without being degraded inside the cells can bind again to other antigens. Accordingly, when the antigen-binding molecule is, for example, an antigen-binding neutralizing molecule, the antigen-binding molecule that easily dissociates from the antigen under the endosomal acidic condition can bind and neutralize antigens multiple times. As a result, antigen-binding molecules whose antigen-binding activity at pH 4.0 to pH 6.5 is lower than that at pH 6.7 to pH 10.0 are superior in retention in the plasma.

In a preferred embodiment, the antigen-binding molecule whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4 includes antigen-binding molecules in which at least one amino acid in the antigen-binding molecule is substituted with histidine or a non-natural amino acid, or in which at least one histidine or a non-natural amino acid has been inserted. The site into which the histidine or non-natural amino acid mutation is introduced is not particularly limited and may be any site, as long as the antigen-binding activity at pH 5.8 is weaker than that at pH 7.4 (the KD(pH5.8)/KD(pH7.4) value is greater or the k_d(pH5.8)/k_d(pH7.4) value is greater) as compared to before substitution. Examples include variable regions and CDRs of an antibody in the case the antigen-binding molecule is an antibody. The number of amino acids to be substituted with histidine or non-natural amino acid and the number of amino acids to be inserted can be appropriately determined by those skilled in the art. One amino acid may be substituted with histidine or non-natural amino acid, or one amino acid may be inserted, or two or more amino acids may be substituted with histidine or non-natural amino acids, or two or more amino acids may be inserted. Moreover, apart from the substitutions to histidine or to non-natural amino acid or insertion of histidine or of non-natural amino acid, deletion, addition, insertion, and/or substitution and such of other amino acids may also be simultaneously carried out. Substitutions to histidine or to non-natural amino acid or insertion of histidine or of non-natural amino acid may be carried out at random using a method such as histidine scanning, which uses histidine instead of alanine in alanine scanning which is known to those skilled in the art. Antigen-binding molecules whose KD(pH5.8)/KD(pH7.4) or k_d(pH5.8)/k_d(pH7.4) is increased as compared to before mutation can be selected from antigen-binding molecules into which histidine or non-natural amino acid mutation has been introduced at random.

Preferred antigen-binding molecules with mutation to histidine or to non-natural amino acid and whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4 include, for example, antigen-binding molecules whose antigen-binding activity at pH 7.4 after the mutation to histidine or to non-natural amino acid is equivalent to the antigen-binding activity at pH 7.4 before the mutation to histidine or to non-natural amino acid. In the present invention, "an antigen-binding molecule after histidine or non-natural amino acid mutation has an antigen-binding activity that is equivalent to that of the antigen-binding molecule before histidine or non-natural amino acid mutation" means that, when the antigen-binding activity of an antigen-binding molecule before histidine or non-natural amino acid mutation is set as 100%, the antigen-binding activity of the antigen-binding molecule after histidine or non-natural amino acid mutation is at least 10% or more, preferably 50% or more, more preferably 80% or more, and still more preferably 90% or more. The antigen-binding activity at pH 7.4 after histidine or non-natural amino acid mutation may be greater than the antigen-binding activity at pH 7.4 before histidine or non-natural amino acid mutation. When the antigen-binding activity of the antigen-binding molecule is decreased due to substitution or insertion of histidine or non-natural amino acid, the antigen-binding activity may be adjusted by introducing substitution, deletion, addition, and/or insertion and such of one or more amino acids into the antigen-binding molecule so that the antigen-binding activity becomes equivalent to that before histidine substitution or insertion. The present invention also includes such antigen-binding molecules whose binding activity has been made equivalent as a result of substitution, deletion, addition, and/or insertion of one or more amino acids after histidine substitution or insertion.

Further, when the antigen-binding molecule is a substance including an antibody constant region, in another preferred embodiment of the antigen-binding molecule whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4, the present invention includes methods for modifying antibody constant regions contained in the antigen-binding molecules. Specific examples of antibody constant regions after modification include the constant regions described in the Examples.

When the antigen-binding activity of the antigen-binding substance at pH 5.8 is weakened compared to that at pH 7.4 (when KD(pH5.8)/KD(pH7.4) value is increased) by the above described methods and such, it is generally preferable that the KD(pH5.8)/KD(pH7.4) value is two times or more, more preferably five times or more, and even more preferably ten times or more as compared to that of the original antibody, but is not particularly limited thereto.

The antigen-binding molecules of the present invention may further have any other property, as long as their antigen-binding activity at pH 4.0 to pH 6.5 is lower than that at pH 6.7 to pH 10.0. For example, the antigen-binding molecules may be agonistic or antagonistic antigen-binding molecules. Preferred antigen-binding molecules of the present invention

include, for example, antagonistic antigen-binding molecules. In general, an antagonistic antigen-binding molecule inhibits receptor-mediated intracellular signaling by inhibiting the binding between a ligand (agonist) and the receptor.

Furthermore, the present invention provides antibodies in which amino acid on at least one site indicated below is substituted with histidine or non-natural amino acid. Amino acid positions are indicated based on the Kabat numbering (Kabat EA *et al.*, (1991) Sequences of Proteins of Immunological Interest, NIH).

Heavy chain: H27, H31, H32, H33, H35, H50, H58, H59, H61, H62, H63, H64, H65, H99, H100b, and H102

Light chain: L24, L27, L28, L32, L53, L54, L56, L90, L92, and L94

Among the above sites, H32, H61, L53, L90, and L94 could be universal modification sites.

When the antigen is the IL-6 receptor (*e.g.*, human IL-6 receptor), preferable modification sites include the following. However, the modification sites are not particularly limited thereto.

Heavy chain: H27, H31, H32, H35, H50, H58, H61, H62, H63, H64, H65, H100b, and H102

Light chain: L24, L27, L28, L32, L53, L56, L90, L92, and L94

When histidine or non-natural amino acid is substituted at multiple sites, preferred combinations of substitution sites include, for example, the combination of H27, H31, and H35; combination of H27, H31, H32, H35, H58, H62, and H102; combination of L32 and L53; and combination of L28, L32, and L53. In addition, preferred combinations of substitution sites of heavy and light chains include the combination of H27, H31, L32, and L53.

When the antigen is IL-6 (*e.g.*, human IL-6), preferable modification sites include the following. However, the modification sites are not particularly limited thereto.

Heavy chain: H32, H59, H61, and H99

Light chain: L53, L54, L90, and L94

When the antigen is the IL-31 receptor (*e.g.*, human IL-31 receptor), preferable modification sites include H33. However, the modification sites are not particularly limited thereto.

The antigen-binding molecules of the present invention may recognize any antigen. Antigens recognized by antibodies of the present invention specifically include the above-mentioned receptor proteins (membrane-bound receptors or soluble receptors), membrane antigens such as cell surface markers, and soluble antigens such as cytokines, for example, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15, IL-31, IL-23, IL-2 receptor, IL-6 receptor, OSM receptor, gp130, IL-5 receptor, CD40, CD4, Fas, osteopontin,

CRTH2, CD26, PDGF-D, CD20, monocyte chemoattractant factor, CD23, TNF-α, HMGB-1, α4 integrin, ICAM-1, CCR2, CD11a, CD3, IFNγ, BLyS, HLA-DR, TGF-β, CD52, and IL-31 receeptor.

Particularly preferred antigens include the IL-6 receptor.

The antigen-binding molecules of the present invention are described above.

In a preferred embodiment of the present invention, the antigen-binding molecules include antibodies. Antibodies having antigen-binding activity and FcRn-binding region include, for example, IgG antibodies. When the antibody used is an IgG antibody, there is no limitation as to its type. It is possible to use IgG1, IgG2, IgG3, IgG4, and such.

The origin of antibody of the present invention is not particularly limited, and may be of any origin. It is possible to use, for example, mouse antibodies, human antibodies, rat antibodies, rabbit antibodies, goat antibodies, camel antibodies, and others. Furthermore, the antibodies may be, for example, the above-described chimeric antibodies, and in particular, modified antibodies with amino acid sequence substitutions, such as humanized antibodies. The antibodies may also be the above-described bispecific antibodies, antibody modification products to which various molecules have been linked, polypeptides including antibody fragments, and antibodies with modified sugar chains.

Generation of chimeric antibodies is known. In the case of a human-mouse chimeric antibody, for example, a DNA encoding an antibody V region may be linked to a DNA encoding a human antibody C region; this can be inserted into an expression vector and introduced into a host to produce the chimeric antibody.

"Humanized antibodies" are also referred to as reshaped human antibodies, and are antibodies in which the complementarity determining region (CDR) of a nonhuman mammal, for example a mouse, is transplanted to the CDR 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). Humanized antibodies can be produced by known methods, for example, the CDR of a mouse antibody can be determined, and a DNA encoding an antibody in which the CDR is linked to the framework region (FR) of a human antibody is obtained. Humanized antibodies can then be produced using a system that uses conventional expression vectors. Such DNAs can be synthesized by PCR, using as primers several oligonucleotides prepared to have portions that overlap with the end regions of both the CDR and FR (see the method described in WO 98/13388). Human antibody FRs linked via CDRs are selected such that the CDRs form a suitable antigen binding site. If required, amino acids in the FRs of an antibody variable region may be substituted so that the CDRs of the reshaped

human antibody can form a suitable antigen binding site (Sato, K. *et al.*, Cancer Res. (1993) 53:10.01-6). Amino acid residues in the FRs that can be modified include portions that directly bind to an antigen via non-covalent bonds (Amit *et al.*, Science (1986) 233: 747-53), portions that influence or have an effect on the CDR structure (Chothia *et al.*, J. Mol. Biol. (1987) 196: 901-17), and portions involved in VH-VL interactions (EP 239400).

When the antibodies of the present invention are chimeric antibodies or humanized antibodies, the C regions of these antibodies are preferably derived from human antibodies. For example, Cγ1, Cγ2, Cγ3, and Cγ4 can be used for the H chain, while Cκ and Cλ can be used for the L chain. Moreover, if required, amino acid mutations may be introduced into the human antibody C region to enhance or lower the binding to Fcγ receptor or FcRn or to improve antibody stability or productivity. A chimeric antibody of the present invention preferably includes a variable region of an antibody derived from a nonhuman mammal and a constant region derived from a human antibody. Meanwhile, a humanized antibody preferably includes CDRs of an antibody derived from a nonhuman mammal and FRs and C regions derived from a human antibody. The constant regions derived from human antibodies preferably include an FcRn-binding region. Such antibodies include, for example, IgGs (IgG1, IgG2, IgG3, and IgG4). The constant regions used for the humanized antibodies of the present invention may be constant regions of antibodies of any isotype. A constant region of human IgG is preferably used, though it is not limited thereto. The FRs derived from a human antibody, which are used for the humanized antibodies, are not particularly limited either, and may be derived from an antibody of any isotype.

The variable and constant regions of chimeric and humanized antibodies of the present invention may be modified by deletion, substitution, insertion, and/or addition, and such, so long as the binding specificity of the original antibodies is exhibited.

Since the immunogenicity in the human body is lowered, chimeric and humanized antibodies using human-derived sequences are thought to be useful when administered to humans for therapeutic purposes or such.

The antibodies of the present invention may be prepared by any method. For example, antibodies whose antigen-binding activity at pH 5.8 is originally greater than or comparable to that at pH 7.4 may be artificially modified through histidine substitution described above or the like so that their antigen-binding activity at pH 5.8 becomes lower than that at pH 7.4. Alternatively, antibodies whose antigen-binding activity at pH 5.8 is lower than that at pH 7.4 may be selected by screening a number of antibodies obtained from an antibody library or hybridomas as described below.

When histidine is substituted for amino acids in an antibody, known sequences may be used for the H chain or L chain amino acid sequence of the antibody before introduction of

histidine mutations, or amino acid sequences of antibodies newly obtained by methods known to those skilled in the art can also be used. For example, the antibodies may be obtained from an antibody library, or they may be obtained by cloning genes encoding antibodies from hybridomas producing monoclonal antibodies.

Regarding antibody libraries, many antibody libraries are already known, and methods for producing antibody libraries are also known; therefore, those skilled in the art can appropriately obtain antibody libraries. For example, regarding antibody phage libraries, one can refer to the literature such as Clackson *et al.*, Nature 1991, 352: 624-8; Marks *et al.*, J. Mol. Biol. 1991, 222: 581-97; Waterhouses *et al.*, Nucleic Acids Res. 1993, 21: 2265-6; Griffiths *et al.*, EMBO J. 1994, 13: 324.0-60; Vaughan *et al.*, Nature Biotechnology 1996, 14: 309-14; and Japanese Patent Kohyo Publication No. (JP-A) H20-504970 (unexamined Japanese national phase publication corresponding to a non-Japanese international publication). In addition, it is possible to use known methods, such as methods using eukaryotic cells as libraries (WO 95/15393) and ribosome display methods. Furthermore, technologies to obtain human antibodies by panning using human antibody libraries are also known. For example, variable regions of human antibodies can be expressed on the surface of phages as single chain antibodies (scFvs) using phage display methods, and phages that bind to antigens can be selected. Genetic analysis of the selected phages can determine the DNA sequences encoding the variable regions of human antibodies that bind to the antigens. Once the DNA sequences of scFvs that bind to the antigens is revealed, suitable expression vectors can be produced based on these sequences to obtain human antibodies. These methods are already well known, and one can refer to WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438, and WO 95/15388.

As for methods for obtaining genes encoding antibodies from hybridomas, known technologies may be basically used, which involve the use of desired antigens or cells expressing the desired antigens as sensitizing antigens, using these to perform immunizations according to conventional immunization methods, fusing the resulting immune cells with known parent cells by conventional cell fusion methods, screening monoclonal antibody producing cells (hybridomas) by conventional screening methods, synthesizing cDNAs of antibody variable regions (V regions) from mRNAs of the obtained hybridomas using reverse transcriptase, and linking them with DNAs encoding the desired antibody constant regions (C regions).

More specifically, sensitizing antigens to obtain the above-described antibody genes encoding the H chains and L chains include both complete antigens with immunogenicity and incomplete antigens including haptens and the like with no antigenicity; however they are not limited to these examples. For example, it is possible to use whole proteins and partial peptides of proteins of interest. In addition, it is known that substances comprising polysaccharides,

nucleic acids, lipids, and such can be antigens. Thus, the antigens of the antibodies of the present invention are not particularly limited. The antigens can be prepared by methods known to those skilled in the art, for example, by baculovirus-based methods (for example, WO 98/46777) and such. Hybridomas can be produced, for example, by the method of Milstein *et al.* (G. Kohler and C. Milstein, Methods Enzymol. 1981, 73: 3-46) and such. When the immunogenicity of an antigen is low, immunization may be performed after linking the antigen with a macromolecule having immunogenicity, such as albumin. Alternatively, if necessary, antigens may be converted into soluble antigens by linking them with other molecules. When transmembrane molecules such as membrane antigens (for example, receptors) are used as antigens, portions of the extracellular regions of the membrane antigens can be used as a fragment, or cells expressing transmembrane molecules on their cell surface may be used as immunogens.

Antibody-producing cells can be obtained by immunizing animals using appropriate sensitizing antigens described above. Alternatively, antibody-producing cells can be prepared by *in vitro* immunization of lymphocytes that can produce antibodies. Various mammals can be used for immunization; such commonly used animals include rodents, lagomorphas, and primates. Such animals include, for example, rodents such as mice, rats, and hamsters; lagomorphas such as rabbits; and primates including monkeys such as cynomolgus monkeys, rhesus monkeys, baboons, and chimpanzees. In addition, transgenic animals carrying human antibody gene repertoires are also known, and human antibodies can be obtained by using these animals (see WO 96/34096; Mendez *et al.*, Nat. Genet. 1997, 15: 146-56). Instead of using such transgenic animals, for example, desired human antibodies having binding activity against antigens can be obtained by *in vitro* sensitization of human lymphocytes with desired antigens or cells expressing the desired antigens, and then fusing the sensitized lymphocytes with human myeloma cells such as U266 (see Japanese Patent Application Kokoku Publication No. (JP-B) H01-59878 (examined, approved Japanese patent application published for opposition)). Furthermore, desired human antibodies can be obtained by immunizing transgenic animals carrying a complete repertoire of human antibody genes, with desired antigens (see WO 93/12227, WO 92/03918, WO 94/02602, WO 96/34096, and WO 96/33735).

Animal immunization can be carried out by appropriately diluting and suspending a sensitizing antigen in phosphate buffered saline (PBS), physiological saline, or such, and mixing it with an adjuvant to emulsify, if necessary. This is then intraperitoneally or subcutaneously injected into animals. Then, the sensitizing antigen mixed with Freund's incomplete adjuvant is preferably administered several times every four to 21 days. Antibody production can be confirmed by measuring the titer of the antibody of interest in animal sera using conventional methods.

Antibody-producing cells obtained from lymphocytes or animals immunized with a desired antigen can be fused with myeloma cells to generate hybridomas using conventional fusing agents (for example, polyethylene glycol) (Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, 1986, 59-103). When required, hybridoma cells can be cultured and grown, and the binding specificity of the antibody produced from these hybridomas can be measured using known analysis methods, such as immunoprecipitation, radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA). Thereafter, hybridomas producing antibodies of interest whose specificity, affinity, or activity has been determined can be subcloned by methods such as limiting dilution.

Next, genes encoding the selected antibodies can be cloned from hybridomas or antibody-producing cells (sensitized lymphocytes, and such) using probes that can specifically bind to the antibodies (for example, oligonucleotides complementary to sequences encoding the antibody constant regions). It is also possible to clone the genes from mRNA using RT-PCR. Immunoglobulins are classified into five different classes, IgA, IgD, IgE, IgG, and IgM. These classes are further divided into several subclasses (isotypes) (for example, IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2; and such). H chains and L chains used in the present invention to produce antibodies are not particularly limited and may originate from antibodies belonging to any of these classes or subclasses; however, IgG is particularly preferred.

Herein, it is possible to modify H-chain-encoding genes and L-chain-encoding genes using genetic engineering technologies. Genetically modified antibodies, such as chimeric antibodies and humanized antibodies, which have been artificially modified for the purpose of decreasing heterologous immunogenicity and such against humans, can be appropriately produced for antibodies such as mouse antibodies, rat antibodies, rabbit antibodies, hamster antibodies, sheep antibodies, and camel antibodies. Chimeric antibodies are antibodies including H chain and L chain variable regions of nonhuman mammal antibody, such as mouse antibody, and the H chain and L chain constant regions of human antibody. Chimeric antibodies can be obtained by ligating a DNA encoding a variable region of a mouse antibody to a DNA encoding a constant region of a human antibody, inserting this into an expression vector, and introducing the vector into a host to produce the antibody. A humanized antibody, which is also called a reshaped human antibody, can be synthesized by PCR using several oligonucleotides produced so that they have overlapping portions at the ends of DNA sequences designed to link the complementarity determining regions (CDRs) of an antibody of a nonhuman mammal such as a mouse. The resulting DNA can be ligated to a DNA encoding a human antibody constant region. The ligated DNA can be inserted into an expression vector, and the vector can be introduced into a host to produce the antibody (see EP 239400 and WO 96/02576). Human antibody FRs that are ligated via the CDR are selected when the CDR forms a favorable

antigen-binding site. If necessary, amino acids in the framework region of an antibody variable region may be substituted such that the CDR of the reshaped human antibody forms an appropriate antigen-binding site (K. Sato *et al.*, Cancer Res. 1993, 53: 10.01-10.06).

In addition to the humanization described above, antibodies may be modified to improve their biological properties, for example, the binding to the antigen. In the present invention, such modifications can be achieved by methods such as site-directed mutagenesis (see for example, Kunkel (1910.0) Proc. Natl. Acad. Sci. USA 82: 488), PCR mutagenesis, and cassette mutagenesis. In general, mutant antibodies whose biological properties have been improved show amino acid sequence homology and/or similarity of 70% or higher, more preferably 80% or higher, and even more preferably 90% or higher (for example, 95% or higher, 97%, 98%, or 99%), when compared to the amino acid sequence of the original antibody variable region. Herein, sequence homology and/or similarity is defined as the ratio of amino acid residues that are homologous (same residue) or similar (amino acid residues classified into the same group based on the general properties of amino acid side chains) to the original antibody residues, after the sequence homology value has been maximized by sequence alignment and gap introduction, if necessary. In general, natural amino acid residues are classified into groups based on the characteristics of their side chains as follows:

(1) hydrophobic: alanine, isoleucine, valine, methionine, and leucine;

(2) neutral hydrophilic: asparagine, glutamine, cysteine, threonine, and serine;

(3) acidic: aspartic acid and glutamic acid;

(4) basic: arginine, histidine, and lysine;

(5) residues that affect the orientation of the chain: glycine, and proline; and

(6) aromatic: tyrosine, tryptophan, and phenylalanine.

In general, a total of six complementarity determining regions (CDRs; hypervariable regions) present on the H chain and L chain variable regions interact with each other to form an antigen-binding site of an antibody. A variable region alone is also known to be capable of recognizing and binding to an antigen, although its affinity is lower than the affinity of the whole binding site. Thus, antibody genes encoding the H chain and L chain of the present invention may encode fragments each including the H chain or L chain antigen-binding site, as long as the polypeptide encoded by the gene retains the activity of binding to the desired antigen.

As described above, the heavy chain variable region is in general constituted by three CDRs and four FRs. In a preferred embodiment of the present invention, amino acid residues to be "modified" can be appropriately selected from amino acid residues, for example, in a CDR or FR. In general, modifications of amino acid residues in the CDRs may reduce the antigen-binding ability. Thus, appropriate amino acid residues to be "modified" in the present invention are preferably selected from amino acid residues in the FRs, but are not limited thereto.

It is possible to select amino acids in a CDR as long as the modification has been confirmed not to reduce the binding ability. Alternatively, by using public databases or such, those skilled in the art can obtain appropriate sequences that can be used as an FR of antibody variable region of an organism such as human or mouse.

Furthermore, the present invention provides genes encoding the antibodies of the present invention. The genes encoding the antibodies of the present invention may be any genes, and may be DNAs, RNAs, nucleic acid analogs, or the like.

Furthermore, the present invention also provides host cells carrying the genes described above. The host cells are not particularly limited and include, for example, *E. coli* and various animal cells. The host cells may be used, for example, as a production system to produce and express the antibodies of the present invention. *In vitro* and *in vivo* production systems are available for polypeptide production systems. Such *in vitro* production systems include, for example, production systems using eukaryotic cells or prokaryotic cells.

Eukaryotic cells that can be used as host cells include, for example, animal cells, plant cells, and fungal cells. Animal cells include: mammalian cells, for example, CHO (J. Exp. Med. (1995) 108: 94.0), COS, HEK293, 3T3, myeloma, BHK (baby hamster kidney), HeLa, and Vero; amphibian cells such as *Xenopus laevis* oocytes (Valle *et al.*, Nature (1981) 291: 338-340); and insect cells such as Sf9, Sf21, and Tn5. CHO-DG44, CHO-DX11B, COS7 cells, HEK293 cells, and BHK cells are preferably used to express the antibodies of the present invention. Among animal cells, CHO cells are particularly preferable for large-scale expression. Vectors can be introduced into host cells, for example, by calcium phosphate methods, DEAE-dextran methods, methods using cationic liposome DOTAP (Boehringer-Mannheim), electroporation methods, and lipofection methods.

Regarding plant cells, for example, *Nicotiana tabacum*-derived cells and duckweed (*Lemna minor*) are known as a protein production system. Calluses can be cultured from these cells to produce the antibodies of the present invention. Regarding fungal cells, known protein expression systems are those using yeast cells, for example, cells of genus *Saccharomyces* (such as *Saccharomyces cerevisiae* and *Saccharomyces pombe*); and cells of filamentous fungi, for example, genus *Aspergillus* (such as *Aspergillus niger*). These cells can be used as a host to produce the antibodies of the present invention.

Bacterial cells can be used in the prokaryotic production systems. Regarding bacterial cells, production systems using *Bacillus subtilis* are known in addition to the production systems using *E. coli* described above. Such systems can be used in producing the antibodies of the present invention.

<Screening methods>

The present invention provides methods of screening for antigen-binding molecules whose antigen-binding activity at acidic pH is lower than that at neutral pH. The present invention also provides methods of screening for antigen-binding molecules which can individually bind to multiple antigens. The present invention also provides methods of screening for antigen-binding molecules which are superior in the retention in plasma. The present invention also provides methods of screening for an antigen-binding molecule that dissociates within a cell from an extracellularly-bound antigen. The present invention also provides methods of screening for an antigen-binding molecule that is bound to an antigen and internalized into a cell, and released to the outside of the cell in an antigen-free form. The present invention also provides methods of screening for an antigen-binding molecule that has increased ability to eliminate antigens in plasma. Furthermore, the present invention also provides methods of screening for antigen-binding molecules which are particularly useful when used as pharmaceutical compositions.

Specifically, the present invention provides methods of screening for antigen-binding molecules, which comprise the steps of:

(a) determining the antigen-binding activity of an antigen-binding molecule at pH 6.7 to pH 10.0;

(b) determining the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5; and

(c) selecting an antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than the antigen-binding activity at pH 4.0 to pH 6.5.

In the screening methods of the present invention, the antigen-binding activity of the antigen-binding molecule at pH 6.7 to pH 10.0 is not particularly limited, as long as it is an antigen-binding activity at a pH between pH 6.7 and pH 10.0. However, for example, a preferred antigen-binding activity is an antigen-binding activity at a pH between pH 7.0 and pH 8.0, and a more preferred antigen-binding activity is an antigen-binding activity at pH 7.4. Further, the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5 is not particularly limited, as long as it is an antigen-binding activity at a pH between pH 4.0 and pH 6.5. However, for example, a preferred antigen-binding activity is an antigen-binding activity at a pH between pH 5.5 to pH 6.5, and a more preferred antigen-binding activity is an antigen-binding activity at pH 5.8 or pH 5.5.

The antigen-binding activity of an antigen-binding molecule can be determined by methods known to those skilled in the art. Conditions other than the pH can be appropriately determined by those skilled in the art. The antigen-binding activity of an antigen-binding molecule can be assessed as dissociation constant (KD), apparent dissociation constant (apparent KD), dissociation rate (k_d), apparent dissociation rate (apparent k_d), or such. These constants

can be determined by methods known to those skilled in the art, for example, using Biacore (GE healthcare), Scatchard plot, or FACS.

Herein, "the step of selecting an antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5" has a same meaning as "the step of selecting an antigen-binding molecule whose antigen-binding activity at pH 4.0 to pH 6.5 is lower than that at pH 6.7 to pH 10.0".

The difference between the antigen-binding activity at pH 6.7 to pH 10.0 and that at pH 4.0 to pH 6.5 is not particularly limited as long as the antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5. However, the antigen-binding activity at pH 6.7 to pH 10.0 is preferably twice or greater, more preferably ten times or greater, and still more preferably 40 times or greater than the antigen-binding activity at pH 4.0 to pH 6.5.

Furthermore, the present invention also provides methods of screening for antigen-binding molecules, which comprise the steps of:

(a) binding an antigen-binding molecule to an antigen under a condition of pH 6.7 to pH 10.0;

(b) placing the antigen-binding molecule that bound to the antigen of (a) under a condition of pH 4.0 to pH 6.5; and

(c) obtaining the antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5.

In addition, the present invention also provides methods of screening for antigen-binding molecules, which comprise the steps of:

(a) selecting an antigen-binding molecule that does not bind to an antigen under a condition of pH 4.0 to pH 6.5;

(b) binding the antigen-binding molecule selected in (a) to an antigen under a condition of pH 6.7 to pH 10.0; and

(c) obtaining the antigen-binding molecule that bound to the antigen under the condition of pH 6.7 to pH 10.0.

Furthermore, the present invention also provides methods of screening for antigen-binding molecules, which comprise the steps of:

(a) binding an antigen-binding molecule to an antigen under a condition of pH 6.7 to pH 10.0;

(b) placing the antigen-binding molecule that bound to the antigen of (a) under a condition of pH 4.0 to pH 6.5;

(c) obtaining the antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5;

(d) amplifying the gene encoding the antigen-binding molecule that dissociated; and

(e) obtaining the eluted antigen-binding molecule.

The steps of (a) to (d) may be repeated twice or more times. Thus, the present invention provides the methods described above further including a step of repeating the steps of (a) to (d) twice or more times. The number for repeating the steps of (a) to (d) is not particularly limited; however, the number is in general ten or less.

Furthermore, the present invention also provides methods of screening for antigen-binding molecules, which comprise the steps of:

(a) selecting an antigen-binding molecule that does not bind to an antigen under a condition of pH 4.0 to pH 6.5;

(b) binding the antigen-binding molecule selected in (a) to an antigen under a condition of pH 6.7 to pH 10.0;

(c) obtaining the antigen-binding molecule that bound to the antigen under the condition of pH 6.7 to pH 10.0;

(d) amplifying the gene encoding the antigen-binding molecule that dissociated; and

(e) collecting the eluted antigen-binding molecule.

The steps of (a) to (d) may be repeated twice or more times. Thus, the present invention provides the methods described above further including a step of repeating the steps of (a) to (d) twice or more times. The number for repeating the steps of (a) to (d) is not particularly limited; however, the number is in general ten or less.

When a phage library or such is used in the screening methods of the present invention, the step of amplifying the gene encoding the antigen-binding molecule can also be a step of amplifying phages.

In the methods of the present invention, binding of the antigen-binding molecule and the antigen may be carried out under any state, without particular limitation. For example, binding of the antigen-binding molecule and the antigen 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, binding of the antigen-binding molecule and the antigen may be carried out by contacting the antigen and antigen-binding molecule in a solution.

Furthermore, the present invention also provides methods of screening for antigen-binding molecules whose binding activity at a first pH is greater than that at a second pH, which comprise the steps of:

(a) binding an antigen-binding molecule to an antigen-immobilized column under the condition of a first pH;

(b) eluting the antigen-binding molecule that had bound to the column at the first pH from the column under the condition of a second pH; and

(c) obtaining the eluted antigen-binding molecule.

Furthermore, the present invention also provides methods of screening for antigen-binding molecules whose binding activity at a first pH is smaller than that at a second pH, which comprise the steps of:

(a) passing an antigen-binding molecule through an antigen-immobilized column under the condition of a first pH;

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

(c) binding the antigen-binding molecule collected in (b) to a column under the condition of a second pH; and

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

Furthermore, the present invention also provides methods of screening for antigen-binding molecules whose binding activity at a first pH is greater than that at a second pH, which comprise the steps of:

(a) binding an antigen-binding molecule library to an antigen-immobilized column under the condition of a first pH;

(b) eluting the antigen-binding molecule from the column under the condition of a second pH;

(c) amplifying the gene encoding the eluted antigen-binding molecule; and

(d) obtaining the eluted antigen-binding molecule.

The steps of (a) to (c) may be repeated twice or more times. Thus, the present invention provides the methods described above further including the step of repeating the steps of (a) to (c) twice or more times. The number for repeating the steps of (a) to (c) is not particularly limited; however, the number is in general ten or less.

In the present invention, each of the first and second pHs may be any pH, as long as they are not identical. In a preferred combination of the first and second pHs, for example, the first pH is between pH 6.7 and pH 10.0, and the second pH is between pH 4.0 and pH 6.5; in a more preferred combination, the first pH is between pH 7.0 and pH 8.0, and the second pH is between pH 5.5 and pH 6.5; and in a still more preferred combination, the first pH is pH 7.4 and the second pH is pH 5.8 or pH5.5.

In another preferred combination of the first and second pHs, for example, the first pH is between pH 4.0 and pH 6.5, and the second pH is between pH 6.7 and pH 10.0; in a more preferred combination, the first pH is between pH 5.5 and pH 6.5, and the second pH is between pH 7.0 and pH 8.0; and in a still more preferred combination, the first pH is pH 5.8 or pH5.5 and the second pH is pH 7.4.

Antigen-binding molecules that are screened by the methods of the present invention may be any antigen-binding molecules. For example, it is possible to use the above-described antigen-binding molecules in the screening of the present invention. For example, it is possible to screen antigen-binding molecules including natural sequences or antigen-binding molecules

including amino acid sequences with substitutions. Preferred antigen-binding molecules that are screened in the present invention include, for example, antigen-binding molecules in which at least one amino acid is substituted with histidine or at least one histidine is inserted. The site of introduction of histidine substitution or insertion is not particularly limited, and may be introduced at any site. Furthermore, histidine substitution or insertion may be introduced at one site, or may be introduced at two or more sites. Furthermore, preferred antigen-binding molecules that are screened in the present invention include, for example, antigen-binding molecules including modified antibody constant regions.

Antigen-binding molecules that are screened by the methods of the present invention may be a number of different antigen-binding molecules introduced with histidine substitutions or insertions at different sites, for example, by histidine scanning.

Thus, the screening methods of the present invention may further comprise the step of substituting at least one amino acid in the antigen-binding molecule with histidine or inserting at least one histidine into the antigen-binding molecule.

In the screening methods of the present invention, non-natural amino acids may be used instead of histidine. Therefore, the present invention can also be understood by replacing the above-mentioned histidine with non-natural amino acids.

Moreover, the screening methods of the present invention may further comprise the step of modifying amino acids of antibody constant regions.

Antigen-binding substances that are screened by the screening methods of the present invention may be prepared by any method. For example, it is possible to use pre-existing antibodies, pre-existing libraries (phage libraries and the like), antibodies and libraries that are prepared from hybridomas obtained by immunizing animals or from B cells of immunized animals, antibodies and libraries (libraries with high content of histidine or non-natural amino acid, libraries introduced with histidine or non-natural amino acid at specific sites, and the like) prepared by introducing histidine mutations or non-natural amino acid mutations into the above-described antibodies and libraries, and so on.

Antigen-binding molecules that bind to the antigen multiple times, which are thus superior in the retention in plasma, can be obtained by the screening methods of the present invention. Thus, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules that are superior in the retention in plasma.

Furthermore, antigen-binding molecules that can bind to the antigen two or more times when administered to animals such as humans, mice, or monkeys can be obtained by the screening methods of the present invention. Thus, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules that can bind to the antigen two or more times.

Furthermore, antigen-binding molecules that are capable of binding to more antigens as compared to the number of their antigen-binding sites when administered to animals such as humans, mice, or monkeys can be obtained by the screening methods of the present invention. Thus, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules that are capable of binding to more antigens as compared to the number of their antigen-binding sites. For example, when the antibody is a neutralizing antibody, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules that can neutralize more antigens as compared to the number of the antigen-binding sites of the antigen-binding molecules.

Furthermore, antigen-binding molecules that are capable of dissociating within a cell from an extracellularly-bound antigen when administered to animals such as humans, mice, or monkeys can be obtained by the screening methods of the present invention. Thus, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules that are capable of dissociating within a cell from an extracellularly-bound antigen.

Furthermore, antigen-binding molecules that are bound to an antigen and internalized into a cell, and released to the outside of the cell in an antigen-free form when administered to animals such as humans, mice, or monkeys can be obtained by the screening methods of the present invention. Thus, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules that are bound to an antigen and internalized into a cell, and released to the outside of the cell in an antigen-free form.

Furthermore, antigen-binding molecules that can rapidly eliminate antigens in plasma when administered to animals such as humans, mice, or monkeys can be obtained by the screening methods of the present invention. Thus, the screening methods of the present invention can be used as screening methods for obtaining antigen-binding molecules with increased (high) ability to eliminate antigens in plasma.

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

In addition, the present invention provides libraries in which the histidine content is increased as compared to the original libraries. Libraries containing antigen-binding molecules with increased histidine content can be used in the screening methods described above and the production methods described hereinafter.

Libraries with increased histidine content can be prepared by methods known to those skilled in the art, which include the following method. 20 types of triplet codons (trinucleotides) encoding 20 types of amino acids can be incorporated at equal frequency when synthesizing nucleic acids to prepare a library by the trinucleotide-method (J Mol Biol. 2008 Feb 29; 376(4): 1182-200). As a result, the position mutated for the library can be made to contain 20 types of amino acids at equal probability. The frequency of histidine in the position mutated for the library can be increased by increasing the proportion of a histidine-encoding trinucleotide as compared to the remaining amino acids among the 20 types in the synthesis.

<Methods for producing antigen-binding molecules>

The present invention provides methods for producing antigen-binding molecules whose antigen-binding activity at the endosomal pH is lower than that at the plasma pH. The present invention also provides methods for producing antigen-binding molecules that are superior in the retention in plasma. The present invention also provides methods for producing antigen-binding molecules that are especially useful when used as pharmaceutical compositions.

Specifically, the present invention provides methods for producing antigen-binding molecules, which comprise the steps of:

(a) determining the antigen-binding activity of an antigen-binding molecule at pH 6.7 to pH 10.0;

(b) determining the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5;

(c) selecting an antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5;

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

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

The present invention also provides methods for producing antigen-binding molecules, which comprise the steps of:

(a) binding an antigen-binding molecule to an antigen at pH 6.7 to pH 10.0;

(b) allowing the antigen-binding molecule bound to the antigen of (a) to stand under the condition of pH 4.0 to pH 6.5;

(c) collecting the antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5;

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

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

Furthermore, the present invention provides methods for producing antigen-binding molecules, which comprise the steps of:

(a) selecting an antigen-binding molecule that does not bind to the antigen under the condition of pH 4.0 to pH 6.5;

(b) binding the antigen under the condition of pH 6.7 to pH 10.0 to the antigen-binding molecule selected in (a);

(c) collecting the antigen-binding molecule that bound to the antigen under the condition of pH 6.7 to pH 10.0;

(d) obtaining the gene encoding the antigen-binding molecule collected in (c); and

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

In addition, the present invention provides methods for producing antigen-binding molecules, which comprise the steps of:

(a) binding an antigen-binding molecule to an antigen under the condition of pH 6.7 to 10.0;

(b) allowing the antigen-binding molecule that bound to the antigen in (a) to stand under the condition of pH 4.0 to pH 6.5;

(c) collecting the antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5;

(d) amplifying the gene encoding the dissociated antigen-binding molecule;

(e) collecting the eluted antigen-binding molecule;

(f) obtaining the gene encoding the antigen-binding molecule collected in (e); and

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

Steps (a) to (d) may be repeated twice or more times. Thus, the present invention provides the methods described above, which further comprise the step of repeating steps (a) to (d) twice or more times. The number of times steps (a) to (d) is repeated is not particularly limited; however, it is generally ten times or less.

Furthermore, the present invention provides methods of screening for antigen-binding molecules, which comprise the steps of:

(a) selecting an antigen-binding molecule that does not bind to the antigen under the condition of pH 4.0 to pH 6.5;

(b) binding the antigen under the condition of pH 6.7 to pH 10.0 to the antigen-binding molecule selected in (a);

(c) collecting the antigen-binding molecule that bound to the antigen under the condition of pH 6.7 to pH 10.0;

(d) amplifying the gene encoding the dissociated antigen-binding molecule;

(e) collecting the eluted antigen-binding molecule;

(f) obtaining the gene encoding the antigen-binding molecule collected in (e); and

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

Steps (a) to (d) may be repeated twice or more times. Thus, the present invention provides the methods described above, which further comprise the step of repeating steps (a) to (d) twice or more times. The number of times steps (a) to (d) is repeated is not particularly limited; however, it is generally ten times or less.

Furthermore, the present invention provides methods for producing antigen-binding molecules whose binding activity at a first pH is greater than that at a second pH, which comprise the steps of:

(a) binding the antigen-binding molecule to a column immobilized with antigen under the first pH condition;

(b) eluting the antigen-binding molecule, which is bound to the column under the first pH condition from the column under a second pH condition;

(c) collecting the eluted antigen-binding molecule;

(d) obtaining the gene encoding the antigen-binding molecule collected in (c); and

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

Furthermore, the present invention provides methods for producing antigen-binding molecules whose binding activity at a first pH is greater than that at a second pH, which comprise the steps of:

(a) binding an antigen-binding molecule library to a column immobilized with antigen under the first pH condition;

(b) eluting the antigen-binding molecule from the column under the second pH condition;

(c) amplifying the gene encoding the eluted antigen-binding molecule;

(d) collecting the eluted antigen-binding molecule;

(e) obtaining the gene encoding the antigen-binding molecule collected in (d); and

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

Steps (a) to (c) may be repeated twice or more times. Thus, the present invention provides the methods described above, which further comprise the step of repeating steps (a) to (c) twice or more times. The number of times steps (a) to (c) is repeated is not particularly limited; however, it is generally ten times or less.

When a phage library or such is used in the production methods of the present invention, the step of amplifying the gene encoding the antigen-binding molecule may be the step of amplifying phages.

Antigen-binding substances that are used in the production methods of the present invention may be prepared by any method. For example, it is possible to use pre-existing antibodies, pre-existing libraries (phage libraries and the like), antibodies and libraries that are prepared from hybridomas obtained by immunizing animals or from B cells of immunized animals, antibodies and libraries (libraries with high content of histidine or non-natural amino

acid, libraries introduced with histidine or non-natural amino acid at specific sites, and the like) prepared by introducing histidine mutations or non-natural amino acid mutations into the above-described antibodies and libraries, and so on.

In the above-described production methods, the antigen-binding activity of the antigen-binding molecule at pH 6.7 to pH 10.0 is not particularly limited, as long as the antigen-binding activity is that at a pH between pH 6.7 and pH 10.0. A preferred antigen-binding activity is that at a pH between pH 7.0 and pH 8.0, and a more preferred antigen-binding activity is that at pH 7.4. Alternatively, the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5 is not particularly limited, as long as the antigen-binding activity is that at a pH between pH 4.0 and pH 6.5. A preferred antigen-binding activity is that at a pH between pH 5.5 to pH 6.5, and a more preferred antigen-binding activity is that at pH 5.8 or pH 5.5.

The antigen-binding activity of an antigen-binding molecule can be determined by methods known to those skilled in the art. Conditions except for pH can be appropriately determined by those skilled in the art.

The step of selecting antigen-binding molecules whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5 is synonymous with the step of selecting antigen-binding molecules whose antigen-binding activity at pH 4.0 to pH 6.5 is lower than that at pH 6.7 to pH 10.0.

The difference in the antigen-binding activity at pH 6.7 to pH 10.0 and at pH 4.0 to pH 6.5 is not particularly limited as long as the antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5. The antigen-binding activity at pH 6.7 to pH 10.0 is preferably twice or greater, more preferably ten times or greater, and still more preferably 40 times or greater than that at pH 4.0 to pH 6.5.

In the production methods described above, the antigen-binding molecule may be bound to the antigen under any condition, and the condition is not particularly limited. For example, the antigen-binding molecule may be bound to the antigen by contacting the antigen with the immobilized antigen-binding molecule, or by contacting the antigen-binding molecule with the immobilized antigen. Alternatively, the antigen-binding molecule may be bound to the antigen by contacting the antigen and antigen-binding molecule in a solution.

In the production methods described above, each of the first and second pHs may be any pH, as long as they are not identical. In a preferred combination of the first and second pHs, for example, the first pH is between pH 6.7 and pH 10.0, and the second pH is between pH 4.0 and pH 6.5; in a more preferred combination, the first pH is between pH 7.0 and pH 8.0, and the second pH is between pH 5.5 and pH 6.5; and in a still more preferred combination, the first pH is pH 7.4 and the second pH is pH 5.8 or pH 5.5.

In another preferred combination of the first and second pHs, for example, the first pH is between pH 4.0 and pH 6.5, and the second pH is between pH 6.7 and pH 10.0; in a more preferred combination, the first pH is between pH 5.5 and pH 6.5, and the second pH is between pH 7.0 and pH 8.0; and in a still more preferred combination, the first pH is pH 5.8 or pH 5.5 and the second pH is pH 7.4.

Antigen-binding molecules that are produced by the production methods described above may be any antigen-binding molecules. Preferred antigen-binding molecules include, for example, antigen-binding molecules in which at least one amino acid is substituted with histidine or at least one histidine has been inserted. The site where such histidine mutation is introduced is not particularly limited and may be introduced at any site. Furthermore, histidine mutation may be introduced at one site or at two or more sites.

Thus, the production methods of the present invention may further comprise the step of substituting at least one amino acid in an antigen-binding molecule with histidine or inserting at least one histidine into antigen-binding molecules.

In the production methods of the present invention, non-natural amino acids may be used instead of histidine. Therefore, the present invention can also be understood by replacing the above-mentioned histidine with non-natural amino acids.

Furthermore, in another embodiment, the antigen-binding molecules that are produced by the production methods described above include, for example, antigen-binding molecules including modified antibody constant regions. Accordingly, the production methods of the present invention may further comprise the step of modifying the amino acids of antibody constant regions.

The antigen-binding molecules that are produced by the production methods of the present invention are superior in the retention in plasma. Thus, the production methods of the present invention can be used as methods for producing antigen-binding molecules that are superior in the retention in plasma.

Furthermore, antigen-binding molecules produced by the production methods are expected to be capable of binding to the antigen two or more times when administered to animals such as humans, mice, or monkeys. Thus, the production methods of the present invention can be used as methods for producing antigen-binding molecules that are capable of binding to the antigen two or more times.

Furthermore, antigen-binding molecules produced by the production methods of the present invention are expected to be capable of binding to more antigens as compared to the number of their antigen-binding sites when administered to animals such as humans, mice, or monkeys. Thus, the production methods of the present invention can be used as methods for

producing antigen-binding molecules that are capable of binding to more antigens as compared to the number of their antigen-binding sites.

Furthermore, antigen-binding molecules produced by the production methods of the present invention are expected to be capable of dissociating within a cell from an extracellularly-bound antigen when administered to animals such as humans, mice, or monkeys. Thus, the production methods of the present invention can be used as methods for producing antigen-binding molecules that are capable of dissociating within a cell from an extracellularly-bound antigen.

Furthermore, antigen-binding molecules produced by the production methods of the present invention are expected to be capable of being bound to an antigen and internalized into a cell as well as being released to the outside of the cell in an antigen-free form, when administered to animals such as humans, mice, or monkeys. Thus, the production methods of the present invention can be used as methods for producing antigen-binding molecules that are capable of being bound to an antigen and internalized into a cell and being released to the outside of the cell in an antigen-free form.

Furthermore, antigen-binding molecules that are produced by the production methods of the present invention are expected to be capable of rapidly eliminating antigens from plasma when administered to animals such as humans, mice, or monkeys. Thus, the production methods of the present invention can be used as method for producing antigen-binding molecules with increased (high) ability to eliminate antigens in plasma.

Furthermore, such antigen-binding molecules can reduce the number of doses in patients and are expected to be especially superior as pharmaceuticals. Thus, the production methods of the present invention can be used as methods for producing antigen-binding molecules for used 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 *Escherichia coli* (*E. coli*) is used as the host, preferred cloning vectors include 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 a body of an organism. For example, pBEST vector (Promega) is preferred for *in vitro* expression; pET vector (Invitrogen) is preferred for *E. coli*; pME18S-FL3 vector (GenBank Accession No. AB009864) is preferred for culture cells; and pME18S vector (Mol Cell Biol. 8:466-472 (1988)) 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, RPMI 1640, 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 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, into the periplasmic space, or into the 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.

<Anti-IL-6 receptor antibodies>

Furthermore, the present invention provides the anti-IL-6 receptor antibodies of (a) to (m) below:

(a) an antibody that includes a heavy chain variable region including an amino acid sequence, in which at least one of Tyr at position 27, Asp at position 31, Asp at position 32, Trp at position 35, Tyr at position 51, Asn at position 59, Ser at position 63, Met at position 106, and Tyr at position 108 in the amino acid sequence of SEQ ID NO: 1 (H53 variable region) has been substituted with His;

(b) an antibody that includes a heavy chain variable region (H3pI) having an amino acid sequence, in which Tyr at position 27, Asp at position 31, and Trp at position 35 in the amino acid sequence of SEQ ID NO: 1 (H53 variable region) have been substituted with His;

(c) an antibody that includes a heavy chain variable region having an amino acid sequence, in which Tyr at position 27, Asp at position 31, Asp at position 32, Trp at position 35, Asn at position 59, Ser at position 63, and Tyr at position 108 in the amino acid sequence of SEQ ID NO: 1 (H53 variable region) have been substituted with His;

(d) an antibody that includes a heavy chain variable region (H170) having an amino acid sequence, in which Tyr at position 27, Asp at position 31, Asp at position 32, Trp at position 35, Asn at position 59, Ser at position 63, and Tyr at position 108 have been substituted with His, and in which Ser at position 99 has been substituted with Val and Thr at position 103 has been substituted with Ile in the amino acid sequence of SEQ ID NO: 1 (H53 variable region);

(e) an antibody that includes a heavy chain variable region having an amino acid sequence, in which Asp at position 31, Tyr at position 51, Ser at position 63, Met at position 106, and Tyr at position 108 in the amino acid sequence of SEQ ID NO: 1 (H53 variable region) have been substituted with His;

(f) an antibody that includes a heavy chain variable region (CLH5) having an amino acid sequence, in which Asp at position 31, Tyr at position 51, Ser at position 63, Met at position 106, and Tyr at position 108 have been substituted with His, and in which Ser at position 99 has been substituted with Phe and Thr at position 103 has been substituted with Ile in the amino acid sequence of SEQ ID NO: 1 (H53 variable region);

(g) an antibody that includes a light chain variable region having an amino acid sequence, in which at least one of Asp at position 28, Tyr at position 32, Glu at position 53, Ser at position 56, and Asn at position 92 in the amino acid sequence of SEQ ID NO: 2 (PF1L variable region) has been substituted with His;

(h) an antibody that includes a light chain variable region (L73) having an amino acid sequence, in which Asp at position 28, Tyr at position 32, and Glu at position 53 in the amino acid sequence of SEQ ID NO: 2 (PF1L variable region) have been substituted with His;

(i) an antibody that includes a light chain variable region (L82) having an amino acid sequence, in which Tyr at position 32 and Glu at position 53 in the amino acid sequence of SEQ ID NO: 1 (H53 variable region) have been substituted with His;

(j) an antibody that includes a light chain variable region (CLL5) having an amino acid sequence, in which Tyr at position 32, Glu at position 53, Ser at position 56, and Asn at position 92 in the amino acid sequence of SEQ ID NO: 2 (PF1L variable region) have been substituted with His;

(k) an antibody that includes the heavy chain variable region of (b) and the light chain variable region of (h);

(l) an antibody that includes the heavy chain variable region of (d) and the light chain variable region of (i); and

(m) an antibody that includes the heavy chain variable region of (f) and the light chain variable region of (h).

Specific examples of the heavy chain variable region having an amino acid sequence in which at least one of Tyr at position 27, Asp at position 31, Asp at position 32, Trp at position 35, Tyr at position 51, Asn at position 59, Ser at position 63, Met at position 106, and Tyr at position 108 in the amino acid sequence of SEQ ID NO: 1 (H53 variable region) has been substituted with His include, for example, the following heavy chain variable regions.

a heavy chain variable region having the amino acid sequence of SEQ ID NO: 3 (H3pI)

a heavy chain variable region having the amino acid sequence of SEQ ID NO: 4 (H170)

a heavy chain variable region having the amino acid sequence of SEQ ID NO: 5 (CLH5)

Specific examples of the light chain variable region having an amino acid sequence in which at least one of Asp at position 28, Tyr at position 32, Glu at position 53, Ser at position 56, and Asn at position 92 in the amino acid sequence of SEQ ID NO: 2 (PF1L variable region) has been substituted with His include, for example, the following light chain variable regions.

a light chain variable region having the amino acid sequence of SEQ ID NO: 6 (L73)

a light chain variable region having the amino acid sequence of SEQ ID NO: 7 (L82)

a light chain variable region having the amino acid sequence of SEQ ID NO: 8 (CLL5)

The amino acid positions and substitutions in each of the above-described antibodies H3pI, H170, CLH5, L73, L82, and CLL5 are shown below in Table 1. The amino acid positions are shown based on the Kabat numbering.

[Table 1]

position	27	31	32	33	35	50	58	61	62	63	64	65	95	99	100B	102
H3pI	H	H		H	H											
H170	H	H	H	H	H		H		H				V	I		H
CLH5		H		H		H			H				F	I	H	H

position	24	27	28	32	53	55	56	90	92	94
L73			H	H	H	H				
L82				H	H	H				
CLL5				H	H	H	H		H	

* In WT, the H chain has histidine at position 33, while the L chain has histidine at position 55.

The present invention provides antibodies comprising at least any one of the amino acid substitutions described above in (a) to (j), and methods for producing the antibodies. Thus, the antibodies of the present invention also include antibodies comprising not only any of the amino acid substitutions described above in (a) to (j) but also amino acid substitution(s) other than those described above in (a) to (j). Amino acid substitutions other than those described above in (a) to (j) include, for example, substitution, deletion, addition, and/or insertion in the amino acid sequence of CDRs and FRs.

Furthermore, the present invention provides the anti-IL-6 receptor antibodies of (1) to (28) below:

(1) an antibody that includes the heavy chain variable region (VH1-IgG1 variable region) having the amino acid sequence from positions 1 to 119 in SEQ ID NO: 21 (VH1-IgG1);

(2) an antibody that includes the heavy chain variable region (VH2-IgG1 variable region) having the amino acid sequence from positions 1 to 119 in SEQ ID NO: 22 (VH2-IgG1);

(3) an antibody that includes the heavy chain variable region (VH3-IgG1 variable region) having the amino acid sequence from positions 1 to 119 in SEQ ID NO: 23 (VH3-IgG1);

(4) an antibody that includes the heavy chain variable region (VH4-IgG1 variable region) having the amino acid sequence from positions 1 to 119 in SEQ ID NO: 24 (VH4-IgG1);

(5) an antibody that includes the light chain variable region (VL1-CK variable region) having the amino acid sequence from positions 1 to 107 in SEQ ID NO: 25 (VL1-CK);

(6) an antibody that includes the light chain variable region (VL2-CK variable region) having the amino acid sequence from positions 1 to 107 in SEQ ID NO: 26 (VL2-CK);

(7) an antibody that includes the light chain variable region (VL3-CK variable region) having the amino acid sequence from positions 1 to 107 in SEQ ID NO: 27 (VL3-CK);

(8) an antibody (Fv1-IgG1) that includes the heavy chain variable region of (2) and the light chain variable region of (6);

(9) an antibody (Fv2-IgG1) that includes the heavy chain variable region of (1) and a light chain variable region having the amino acid sequence of SEQ ID NO: 7 (L82);

(10) an antibody (Fv3-IgG1) that includes the heavy chain variable region of (4) and the light chain variable region of (5);

(11) an antibody (Fv4-IgG1) that includes the heavy chain variable region of (3) and the light chain variable region of (7);

(12) an antibody (VH3-IgG2ΔGK) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 33;

(13) an antibody (VH3-M58) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 34;

(14) an antibody (VH3-M73) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 35;

(15) an antibody (Fv4-IgG2ΔGK) that includes the heavy chain of (12) and a light chain having the amino acid sequence of SEQ ID NO: 27 (VL3-CK);

(16) an antibody (Fv4-M58) that includes the heavy chain of (13) and a light chain having the amino acid sequence of SEQ ID NO: 27 (VL3-CK);

(17) an antibody (Fv4-M73) that includes the heavy chain of (14) and a light chain having the amino acid sequence of SEQ ID NO: 27 (VL3-CK);

(18) an antibody (VH2-M71) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 36 (VH2-M71);

(19) an antibody (VH2-M73) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 37 (VH2-M73);

(20) an antibody (VH4-M71) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 38 (VH4-M71);

(21) an antibody (VH4-M73) that includes a heavy chain having the amino acid sequence of SEQ ID NO: 39 (VH4-M73);

(22) an antibody (Fv1-M71) that includes the heavy chain of (18) and a light chain having the amino acid sequence of SEQ ID NO: 26 (VL2-CK);

(23) an antibody (Fv1-M73) that includes the heavy chain of (19) and a light chain having the amino acid sequence of SEQ ID NO: 26 (VL2-CK);

(24) an antibody (Fv3-M71) that includes the heavy chain of (20) and a light chain having the amino acid sequence of SEQ ID NO: 25 (VL1-CK);

(25) an antibody (Fv3-M73) that includes the heavy chain of (21) and a light chain having the amino acid sequence of SEQ ID NO: 25 (VL1-CK);

(26) an antibody that includes a light chain having the amino acid sequence of SEQ ID NO: 25 (VL1-CK);

(27) an antibody that includes a light chain having the amino acid sequence of SEQ ID NO: 26 (VL2-CK); and

(28) an antibody that includes a light chain having the amino acid sequence of SEQ ID NO: 27 (VL3-CK).

Furthermore, the present invention provides the FRs and CDRs of (a) to (v) below:

(a) the heavy chain CDR1 of SEQ ID NO: 40 (VH1, 2, 3, 4);

(b) the heavy chain CDR2 of SEQ ID NO: 41 (VH1, 2);

(c) the heavy chain CDR2 of SEQ ID NO: 42 (VH3);

(d) the heavy chain CDR2 of SEQ ID NO: 43 (VH4);

(e) the heavy chain CDR3 of SEQ ID NO: 44 (VH1, 2);

(f) the heavy chain CDR3 of SEQ ID NO: 45 (VH3, 4);

(g) the heavy chain FR1 of SEQ ID NO: 46 (VH1, 2);

(h) the heavy chain FR1 of SEQ ID NO: 47 (VH3, 4):

(i) the heavy chain FR2 of SEQ ID NO: 48 (VH1, 2, 3, 4);

(j) the heavy chain FR3 of SEQ ID NO: 49 (VH1);

(k) the heavy chain FR3 of SEQ ID NO: 50 (VH2);

(l) the heavy chain FR3 of SEQ ID NO: 51 (VH3, 4);

(m) the heavy chain FR4 of SEQ ID NO: 52 (VH1, 2, 3, 4);

(n) the light chain CDR1 of SEQ ID NO: 53 (VL1, 2);

(o) the light chain CDR1 of SEQ ID NO: 54 (VL3);

(p) the light chain CDR2 of SEQ ID NO: 55 (VL1, VL3);

(q) the light chain CDR2 of SEQ ID NO: 56 (VL2);

(r) the light chain CDR3 of SEQ ID NO: 57 (VL1, 2, 3);

(s) the light chain FR1 of SEQ ID NO: 58 (VL1, 2, 3);

(t) the light chain FR2 of SEQ ID NO: 59 (VL1, 2, 3);

(u) the light chain FR3 of SEQ ID NO: 60 (VL1, 2, 3); and

(v) the light chain FR4 of SEQ ID NO: 61 (VL1, 2, 3).

The respective sequences of the above (a) to (v) are shown in Fig. 25. Furthermore, the present invention provides polypeptides including any one of the FRs and CDRs of the above (a) to (v).

The anti-IL-6 receptor antibodies of the present invention also include fragments and modified products of antibodies including any of the amino acid substitutions described above. Such antibody fragments include, for example, Fab, F(ab')2, Fv, single chain Fv (scFv) in which Fv of H and L chains are linked together via an appropriate linker, single domain H chain and single domain L chain (for example, Nat. Biotechnol. 2005 Sep;23(9):1126-36), Unibody (WO 2007059782 A1), and SMIP (WO 2007014278 A2). The origin of antibodies is not particularly limited. The antibodies include human, mouse, rat, and rabbit antibodies. The antibodies of the present invention may also be chimeric, humanized, fully humanized antibodies, or such.

Specifically, such antibody fragments are obtained by treating antibodies with enzymes, for example, papain or pepsin, or by constructing genes that encode such antibody fragments, inserting them into expression vectors, and then expressing them in appropriate host cells (see, for example, Co, M.S. *et al.*, J. Immunol. (1994) 152, 2968-2976; Better, M. & Horwitz, A. H. Methods in Enzymology (1989) 178, 476-496; Plueckthun, A. & Skerra, A. Methods in Enzymology (1989) 178, 497-515; Lamoyi, E., Methods in Enzymology (1989) 121, 652-663; Rousseaux, J. *et al.*, Methods in Enzymology (1989) 121, 663-66; Bird, R. E. *et al.*, TIBTECH (1991) 9, 132-137).

The present invention provides methods of producing (i) a polypeptide of the present invention, or (ii) a polypeptide encoded by a gene encoding the polypeptide of the present invention, wherein the methods comprise the step of culturing a host cell comprising a vector into which a polynucleotide encoding the polypeptide of the present invention is introduced.

More specifically, the present invention provides methods of producing a polypeptide of the present invention, which comprise the steps of:

(a) culturing a host cell comprising a vector into which a gene encoding the polypeptide of the present invention is introduced; and

(b) obtaining the polypeptide encoded by the gene.

scFv is obtained by linking the V regions of antibody H and L chains. In such scFv, the H chain V region is linked to the L chain V region via a linker, preferably a peptide linker (Huston, J. S. *et al.*, Proc. Natl. Acad. Sci. U.S.A. (1988) 10.0, 5879-5883). The H chain and L chain V regions in an scFv may be derived from any of the antibodies described above. The peptide linker to link the V regions includes, for example, arbitrary single chain peptides of 12 to 19 amino acid residues.

When an anti-IL-6 receptor antibody of the present invention includes a constant region, the constant region may be of any type, for example, IgG1, IgG2, or IgG4 may be used. The constant region is preferably a human antibody constant region. Alternatively, the constant region may be a modified form including substitution, deletion, addition, and/or insertion in the amino acid sequence of human IgG1, human IgG2, or human IgG4 constant regions.

Preferred IL-6 receptor to which an anti-IL-6 receptor antibody of the present invention binds is human IL-6 receptor.

The anti-IL-6 receptor antibodies of the present invention are superior in the retention in plasma, and they exist for a prolonged period in the plasma in a form capable of binding to the antigen, i.e., soluble or membrane-associated IL-6 receptors. Thus, the anti-IL-6 receptor antibodies bind *in vivo* to soluble or membrane-associated IL-6 receptors for a prolonged period. Furthermore, the anti-IL-6 receptor antibodies are capable of binding to IL-6 receptors twice or more times, and thus assumed to be able to neutralize three or more IL-6 receptors.

<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. The antigen-binding molecules of the present invention and antigen-binding molecules produced by the production methods of the present invention are superior in the retention in plasma, and thus, expected to reduce the administration frequency of the antigen-binding molecules, and are therefore useful as pharmaceutical compositions. The pharmaceutical composition of the present invention may include pharmaceutically acceptable carriers.

In the present invention, pharmaceutical compositions ordinarily 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 pharmaceutically 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 1000 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.

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 (Gln) residue into a pyroglutamic acid (pGlu) residue 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.

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 Examples, but it is not to be construed as being limited thereto.

[Example 1] Production of Modified Humanized PM1 Antibody

Preparation of Recombinant Soluble Human IL-6 Receptor (SR344)

A recombinant human IL-6 receptor of the human IL-6 receptor, which served an antigen, was prepared as described below. A CHO cell line that constantly expresses a soluble human IL-6 receptor (hereinafter referred to as SR344) (Yamasaki, et al., Science 1988; 241: 825-828 (GenBank #X12830)) consisting of the amino acid sequence from the 1st amino acid to the 344th amino acid on the N-terminal side as reported in J. Biochem., 108, 673-676 (1990), was produced.

SR344 was purified from culture supernatant obtained from the SR344-expressing CHO cells using three column chromatographies: Blue Sepharose 6 FF column chromatography, affinity chromatography using a column in which an antibody specific to SR344 is immobilized, and gel filtration column chromatography. The fraction that eluted as the main peak was used as the final purified product.

Preparation of Recombinant Cynomolgus Monkey Soluble IL-6 Receptor (cIL-6R)

Oligo DNA primers Rhe6Rf1 (SEQ ID NO: 16) and Rhe6Rr2 (SEQ ID NO: 17) were produced based on the publicly-available rhesus monkey IL-6 receptor gene sequence (Birney et al., Ensemble 2006, Nucleic Acids Res., 2006, Jan. 1; 34 (Database issue): D556-61). Using cDNA prepared from cynomolgus pancreas as a template, a DNA fragment encoding the entire length of cynomolgus monkey IL-6 receptor gene was prepared by PCR using primers Rhe6Rf1 and Rhe6Rr2. Using the resulting DNA fragment as a template, a 1131 bp DNA fragment (SEQ ID NO: 20) encoding a protein in which 6xHis is added to the C terminal of the soluble region (Met1-Pro363) containing a signal region of cynomolgus monkey IL-6 receptor gene, was amplified by PCR using the oligo DNA primers CynoIL6R N-EcoRI (SEQ ID NO: 18) and CynoIL6R C-NotI-His (SEQ ID NO: 19). The resulting DNA fragment was digested with EcoRI and NotI and inserted into a mammalian cell expression vector, and this was then used to produce a stable expression CHO line (cyno.sIL-6R-producing CHO cells).

A culture medium of cyno.sIL-6R-producing CHO cells was purified with a HisTrap column (GE Healthcare Biosciences), concentrated using Amicon Ultra-15 Ultracel-10k (Millipore), and further purified with a Superdex 200 pg 16/60 gel filtration column (GE Healthcare Biosciences) to obtain the final purified product of soluble cynomolgus monkey IL-6 receptor (hereinafter referred to as cIL-6R).

Preparation of Recombinant Cynomolgus Monkey IL-6 (cIL-6)

Cynomolgus monkey IL-6 was prepared as follows. A nucleotide sequence encoding the 212 amino acids registered under SWISSPROT Accession No. P79341 was produced, cloned into a mammalian cell expression vector, and introduced into CHO cells to produce a stable expression cell line (cyno.IL-6-producing CHO cells). A culture medium of cyno.IL-6-producing CHO cells was purified with an SP-Sepharose/FF column (GE Healthcare Biosciences), concentrated using Amicon Ultra-15 Ultracel-5k (Millipore), and then further purified with a Superdex 75 pg 26/60 gel filtration column (GE Healthcare Biosciences). This was concentrated using Amicon Ultra-15 Ultracel-5k (Millipore) to obtain a final purified product of cynomolgus monkey IL-6 (hereinafter referred to as cIL-6).

Establishment of Human gp130-Expressing BaF3 Cell Line

A BaF3 cell line expressing human gp130 was established as indicated below in order to obtain a cell line exhibiting IL-6-dependent growth.

Full-length human gp130 cDNA (Hibi et al., Cell 1990; 63: 1149-1157 (GenBank #NM_002184)) was amplified by PCR and cloned into the expression vector pCOS2Zeo, which was prepared by removing the DHFR gene expression site from pCHOI (Hirata, et al., FEBS

Letter 1994; 356: 244-248) and inserting a Zeocin resistance gene expression site, to construct pCOS2Zeo/gp130. Full-length human IL-6R cDNA was amplified by PCR and cloned into pcDNA3.1(+) (Invitrogen) to construct hIL-6R/pcDNA3.1(+). 10 μg of pCOS2Zeo/gp130 was mixed into BaF3 cells (0.8×10^7 cells) suspended in PBS, and pulsed using a Gene Pulser (Bio-Rad) at a voltage of 0.33 kV and capacitance of 950 μFD. BaF3 cells having undergone gene introduction by electroporation treatment were cultured one whole day and night in RPMI1640 medium (Invitrogen) containing 0.2 ng/mL of mouse interleukin-3 (Peprotech) and 10% fetal bovine serum (hereinafter referred to as FBS, HyClone), and then screened by adding RPMI1640 medium containing 100 ng/mL of human interleukin-6 (R&D Systems), 100 ng/mL of human interleukin-6 soluble receptor (R&D Systems) and 10% FBS to establish a human gp130-expressing BaF3 cell line (hereinafter referred to as BaF3/gp130). Since this BaF3/gp130 proliferates in the presence of human interleukin-6 (R&D Systems) and SR344, it can be used to evaluate the growth inhibitory activity (namely, IL-6 receptor-neutralizing activity) of anti-IL-6 receptor antibody.

Production of Humanized Anti-IL-6 Receptor Antibody

In the context of the instant example and elsewhere herein, the term "wild type" is abbreviated as WT, the term "wild type H chain" is abbreviated as H(WT) (amino acid sequence of SEQ ID NO: 9), and the term "wild type L chain is abbreviated as L(WT) (amino acid sequence: SEQ ID NO. 10). In this context, mutations were introduced into the framework sequence and CDR sequence of humanized mouse PM1 antibody described in Cancer Res. 1993, Feb. 15; 53(4): 851-6, to produce modified H chains H53 (amino acid sequence: SEQ ID NO: 1) and PF1H (amino acid sequence: SEQ ID NO: 11), and modified L chains L28 (amino acid sequence: SEQ ID NO: 12) and PF1L (amino acid sequence: SEQ ID NO: 2). More specifically, the mutants were produced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the method described in the instructions provided, and the resulting plasmid fragments were inserted into a mammalian cell expression vector to produce the desired H chain expression vectors and L chain expression vectors. The nucleotide sequences of the obtained expression vectors were determined using conventional methodologies known to persons skilled in the art.

Expression and Purification of Humanized Anti-IL-6 Receptor Antibody

The antibodies were expressed by the method described below. 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 6×10^5

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 2000 g, 5 min, room temperature) to remove cells, and sterilized by filtering through 0.22-μm filter MILLEX®-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 PACE method (Protein Science 1995; 4:2411-2423).

[Example 2] Production of pH-Dependently-Binding Antibody H3pI/L73

Method for Creating Antibody Capable of Neutralizing Antigen Multiple Times

Since IgG molecules are divalent, a single IgG molecule can neutralize up to two antigen molecules when the two sites bind to the antigens; however, it cannot neutralize three or more antigen molecules. Therefore, to maintain the neutralizing effect of a neutralizing antibody over a certain period, it is necessary to administer an amount of the antibody equal to or greater than the amount of antigen produced during the period. Thus, there is a limitation on the extent to which the required dose of antibody can be reduced by improving the pharmacokinetics or affinity of antibody. Therefore, if it were possible to neutralize two or more antigen molecules with a single IgG molecule, the same dose could improve the duration of neutralizing effect, or alternatively the dose of antibody required to achieve the same duration could be reduced.

For neutralizing antibodies, there are two types of target antigens: soluble-type antigens, which are present in plasma, and membrane-bound antigens, which are expressed on the surface of cells.

When the antigen is a membrane-bound antigen, an administered antibody binds to the membrane antigen on the cellular surface, and the antibody is subsequently taken up into endosomes within the cell by internalization together with the membrane antigen bound to the antibody. Then, the antibody which is kept bound to the antigen moves to a lysosome where it is degraded by lysosome together with the antigen. The elimination of antibody from the plasma mediated by internalization by membrane antigen is referred to as antigen-dependent elimination, and this has been reported for numerous antibody molecules (Drug Discov. Today, 2006 Jan; 11(1-2): 81-8). Since a single IgG antibody molecule binds to two antigen molecules

when it divalently binds to antigens, and is then internalized and directly degraded by lysosome, a single ordinary IgG antibody cannot neutralize two or more antigen molecules (Fig. 1).

The reason for the long retention (slow elimination) of IgG molecules in plasma is that FcRn, known as an IgG molecule salvage receptor, functions (Nat. Rev. Immunol. 2007 Sep; 7(9): 715-25). IgG molecules that have been taken up into endosomes by pinocytosis bind to FcRn expressed in endosomes under intraendosomal acidic conditions. IgG molecules bound to FcRn move to the cell surface where they dissociate from FcRn under neutral conditions in plasma and return to plasma, while IgG molecules unable to bind to FcRn proceed into lysosomes where they are degraded (Fig. 2).

IgG molecules bound to a membrane antigen are taken up into intracellular endosomes by internalization, move into lysosomes while bound to the antigen, and undergo degradation. When an IgG antibody divalently binds to antigens, it neutralizes two antigen molecules and undergoes degradation together with the antigens. If the IgG antibody, when taken up into intracellular endosomes by internalization, can dissociate from the antigen under intraendosomal acidic conditions, the dissociated antibody may be able to bind to FcRn expressed in the endosomes. The IgG molecule dissociated from the antigen and bound to FcRn is transferred to the cell surface and then dissociated from FcRn under neutral conditions in the plasma, thereby return to the plasma again. The IgG molecule that has returned to the plasma is able to bind to a new membrane antigen again. The repetition of this process allows a single IgG molecule to repeatedly bind to membrane antigens, thereby enabling neutralization of a multiple antigens with a single IgG molecule (Fig. 3).

In the case of a soluble antigen, an antibody administered binds to the antigen in the plasma, and remains in the plasma in the form of an antigen-antibody complex. Normally, while the retention of antibody in plasma is very long (elimination rate is very slow) due to the function of FcRn as described above, the retention of antigen in plasma is short (elimination rate is fast). Thus, antibody-bound antigens show retention in plasma comparable to that of antibody (elimination rate is very slow). Antigens are produced in the body at a constant rate and, in the absence of antibody, present in plasma at a concentration at which the antigen production rate and the antigen elimination rate are under equilibrium. In the presence of antibody, most of the antigens are bound to antibodies, resulting in the very slow elimination of antigens. Thus, the antigen concentration in plasma increases as compared with that in the absence of antibody (Kidney Int. 2003, 64, 697-703; J. National Cancer Institute 2002, 94(19), 1484-1493; J. Allergy and Clinical Immunology 1997, 100(1), 110-121; Eur. J. Immunol. 1993, 23; 2026-2029). Even if the affinity of antibody for antigen is infinite, antigen concentration elevates as antibody is slowly eliminated from the plasma, and the neutralizing effect of antibody terminates after the concentrations of antibody and antigen become equal. Although antibodies

with a stronger dissociation constant (KD) can neutralize soluble antigens at a lower antibody concentration, antibodies at a concentration half or less than the concentration of antigen present are unable to neutralize antigens regardless of how strong the affinity of antibody is (Biochem. Biophys. Res. Commun. 2005 Sep 9; 334(4): 1004-13). As is the case with IgG molecules not bound to antigens, IgG molecules bound to antigens in the plasma are also taken up into endosomes by pinocytosis, and bind to FcRn expressed in endosomes under intraendosomal acidic conditions. The IgG molecules bound to FcRn moves to the cell surface while the antibody is kept bound to the antigen and then dissociate from the FcRn under neutral conditions in the plasma. Since the IgG molecules return to the plasma while bound to the antigen, they cannot bind to new antigens in the plasma. In this case, if IgG molecules can dissociate from the antigen under intraendosomal acidic conditions, the dissociated antigen will not be able to bind to FcRn and thereby may be degraded by lysosomes. On the other hand, the IgG molecules can return to the plasma again by binding to FcRn. Since the IgG molecules that have returned to the plasma have already dissociated from the antigen in endosomes, they are able to bind to a new antigen again in the plasma. The repetition of this process allows a single IgG molecule to repeatedly bind to soluble antigens. This enables a single IgG molecule to neutralize multiple antigens (Fig. 4).

Thus, regardless of whether the antigen is a membrane antigen or soluble antigen, if the dissociation of IgG antibody from the antigen is possible under intraendosomal acidic conditions, a single IgG molecule would be able to repeatedly neutralize antigens. In order for IgG antibodies to dissociate from antigens under intraendosomal acidic conditions, it is necessary that antigen-antibody binding be considerably weaker under acidic conditions than under neutral conditions. Since membrane antigens on the cell surface need to be neutralized, antibodies have to strongly bind to antigens at the cell surface pH, namely pH 7.4. Since the intraendosomal pH has been reported to be typically pH 5.5 to 6.0 (Nat. Rev. Mol. Cell. Biol. 2004 Feb; 5(2): 121-32), an antibody that weakly binds to an antigen at pH 5.5 to 6.0 is considered to dissociate from the antigen under intraendosomal acidic conditions. More specifically, a single IgG molecule that strongly binds to an antibody at the cell surface pH of 7.4 and weakly binds to the antigen at the intraendosomal pH of 5.5 to 6.0 may be able to neutralize a multiple antigens and thereby improve the pharmacokinetics.

In general, protein-protein interactions consist of hydrophobic interaction, electrostatic interaction and hydrogen bonding, and the binding strength is typically expressed as a binding constant (affinity) or apparent binding constant (avidity). pH-dependent binding, whose binding strength varies between neutral conditions (pH 7.4) and acidic conditions (pH 5.5 to 6.0), is present in naturally-occurring protein-protein interactions. For example, the above-mentioned binding between IgG molecules and FcRn known as a salvage receptor for IgG

molecules is strong under acidic conditions (pH 5.5 to 6.0) but remarkably weak under neutral conditions (pH 7.4). Most of such pH-dependently changing protein-protein interactions are associated with histidine residues. Since the pKa of histidine residue is in the vicinity of pH 6.0 to 6.5, the proton dissociation state of histidine residues varies between neutral conditions (pH 7.4) and acidic conditions (pH 5.5 to 6.0). Specifically, histidine residues are not charged and function as hydrogen atom acceptors under neutral conditions (pH 7.4), while they become positively charged and function as hydrogen atom donors under acidic conditions (pH 5.5 to 6.0). It has been reported that the pH-dependent binding of the above-described IgG-FcRn interaction is also associated with histidine residues present in IgG (Mol. Cell. 2001 Apr; 7(4): 867-77).

Therefore, pH-dependence can be imparted to protein-protein interactions by substituting an amino acid residue involved in protein-protein interactions with a histidine residue, or by introducing a histidine into an interaction site. Such attempts have also been made in protein-protein interactions between antibodies and antigens, and a mutant antibody with antigen-binding ability decreased under acidic conditions has been successfully acquired by introducing histidine into the CDR sequence of an anti-egg white lysozyme antibody (FEBS Letter (vol. 309, No. 1, 85-88, 1992)). In addition, an antibody that is prepared by introducing histidine into its CDR sequence and specifically binds to an antigen at the low pH of cancer tissues but weakly binds under neutral conditions has been reported (WO 2003-105757).

Although methods for introducing pH dependency into antigen-antibody reactions have been reported as described above, an IgG molecule that neutralizes multiple antigens by strongly binding to antigens at the body fluid pH of 7.4 but weakly binding to antigens at the intraendosomal pH of pH 5.5 to 6.0 has not been reported. In other words, there have been no reports relating to modifications that significantly reduce the binding under acidic conditions while maintaining the binding under neutral conditions such that, as compared to an unmodified antibody, a modified antibody binds to antigens multiple times *in vivo* and thereby shows improved pharmacokinetics as well as improved duration of the neutralizing effect at the same dose.

The IL-6 receptor is present in the body in the form of either soluble IL-6 receptor or membrane IL-6 receptor (Nat. Clin. Pract. Rheumatol. 2006 Nov; 2(11): 619-26). Anti-IL-6 receptor antibodies bind to both the soluble IL-6 receptor and membrane IL-6 receptor, and neutralize their biological action. It is considered that, after binding to the membrane IL-6 receptor, anti-IL-6 receptor antibodies are taken up into intracellular endosomes by internalization while bound to the membrane IL-6 receptor, then move into lysosomes while the antibodies are kept bound to the membrane IL-6 receptor, and undergo degradation by lysosomes together with the membrane IL-6 receptor. In fact, it has been reported that a humanized anti-IL-6 receptor antibody exhibits non-linear clearance, and its antigen-dependent elimination

greatly contributes to the elimination of the humanized anti-IL-6 receptor antibody (The Journal of Rheumatology, 2003, 30; 71426-1435). Thus, one humanized anti-IL-6 receptor antibody binds to one or two membrane IL-6 receptors (monovalently or divalently), and is then internalized and degraded in lysosomes. Therefore, if it is possible to produce modified antibodies that exhibit greatly reduced binding ability under acidic conditions but retain the same binding ability as the wild type humanized anti-IL-6 receptor antibody under neutral conditions (pH-dependent binding anti-IL-6 receptor antibody), multiple IL-6 receptors can be neutralized with a single humanized anti-IL-6 receptor antibody. Thus, in comparison with wild type humanized anti-IL-6 receptor antibodies, pH-dependent binding anti-IL-6 receptor antibodies may improve the duration of the neutralizing effect *in vivo* at the same dosage.

Production of pH-Dependently Binding Humanized Anti-IL-6 Receptor Antibody H3pI/L73:

Introduction of histidine into a CDR has been reported as a method for introducing pH-dependent binding to antigen-antibody reaction (FEBS Letter (vol. 309, No. 1, 85-88, 1992)). In order to find amino acid residues exposed on the surface of the variable region of the H53/PF1L produced in Example 1 and possible residues interacting with the antigen, a Fv region model of H53/PF1L was created by homology modeling using MOE software (Chemical Computing Group Inc.). A three-dimensional model constructed on the basis of the sequence information of H53/PF1L was used to select H27, H31, H35, L28, L32 and L53 (Kabat numbering, Kabat, E.A. et al., 1991, Sequences of Proteins of Immunological Interest, NIH) as mutation sites that may introduce pH-dependent antigen-binding by histidine introduction. The product in which the residues at H27, H31 and H35 in H53 produced in Example 1 were substituted with histidines was designated as H3pI (amino acid sequence: SEQ ID NO: 3), and the product in which the residues at L28, L32 and L53 in PF1L produced in Example 1 were substituted with histidines was designated as L73 (amino acid sequence: SEQ ID NO: 6).

Production, Expression and Purification of H3pI/L73 Expression Vector

Amino acid modification was carried out to produce antibodies modified at the selected sites. Mutations were introduced into H53 (nucleotide sequence: SEQ ID NO: 13) and PF1L (nucleotide sequence: SEQ ID NO: 14) produced in Example 1 to produce H3pI (amino acid sequence: SEQ ID NO: 3) and L73 (amino acid sequence: SEQ ID NO: 6). More specifically, the QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used according to the method described in the instructions provided, and the resulting plasmid fragments were inserted into a mammalian cell expression vector to produce the desired H chain expression vector and L chain expression vector. The nucleotide sequences of the resulting expression vectors were determined using a method known to persons skilled in the art. H3pI/L73 which uses H3pI for

the H chain and L73 for the L chain was expressed and purified by the method described in Example 1.

[Example 3] Conferring pH-Dependent Antigen Binding Ability by His Modification of CDR Using Phage Display Technology

Production of scFv Molecule of Humanized PM1 Antibody

The humanized PM1 antibody, which is a humanized anti-IL-6R antibody (Cancer Res. 1993 Feb 15; 53(4): 851-6), was converted into scFv. The VH and VL regions were amplified by PCR, and humanized PM1 HL scFv having the linker sequence GGGGSGGGGSGGGGS (SEQ ID NO. 15) between VH and VL was produced.

Selection of Histidine-Introducible Positions by Histidine Scanning

PCR was performed using the produced humanized PM1 HL scFv DNA as a template to produce a histidine library in which any one of the CDR amino acids is replaced with histidine. The library portions were constructed by PCR using primers in which the codon of an amino acid desired to be mutated for the library was replaced with CAT, a codon corresponding to histidine, and other portions were constructed by normal PCR. These portions were then linked by assemble PCR. The constructed library was digested with SfiI, inserted into a phagemide vector pELBG lacI that was also digested with SfiI, and then used to transform XL1-Blue (Stratagene). The resulting colonies were used to evaluate antigen binding by phage ELISA and analyze the sequence of HL scFv. Phage ELISA was carried out using a plate coated with SR344 at 1 μg/mL in accordance with J. Mol. Biol. 1992; 227: 381-388. Clones that were found to bind to SR344 were subjected to sequence analysis using specific primers.

Phage titer was determined by ELISA with an anti-Etag antibody (GE Healthcare) and anti-M13 antibody (GE Healthcare). This value was then used to select positions where substitution of the CDR residue with histidine did not significantly alter the binding ability as compared to humanized PM1 HL scFv, based on the results of phage ELISA for SR344. The selected positions are shown in Table 2. Numbering of each residue was in accordance with the Kabat numbering (Kabat, et al., 1991, Sequences of Proteins of Immunological Interest, NIH).

[Table 2] Positions of Histidine Substitution Not Significantly Affecting Binding Ability
H31, H50, H54, H56, H57, H58, H59, H60, H61, H62, H63, H64, H65, H100a, H100b, H102
L24, L26, L27, L28, L30, L31, L32, L52, L53, L54, L56, L90, L92, L93, L94

Construction of Histidine-Modified CDR Library

A library was designed in which the amino acids of CDR residues that did not significantly alter the binding ability when substituted with histidine as shown in Table 2 (histidine-introducible positions) are their original sequence (wild type sequence) or histidine. The library was constructed based on the sequences of the H chain PF1H and the L chain PF1L produced in Example 1 such that the mutated positions for the library have the original sequences or histidines (either the original sequence or histidines).

The library portions were constructed by PCR using primers that were designed such that a position desired to be mutated for the library has the original amino acid codon or histidine codon, and other portions were produced by normal PCR, or by PCR using synthetic primers as in the library portions. These portions were then linked by assemble PCR (J. Mol. Biol. 1996; 256: 77-88).

This library was used to construct a ribosome display library in accordance with J. Immunological Methods 1999; 231: 119-135. In order to carry out *Escherichia coli* cell-free *in vitro* translation, an SDA sequence (ribosome binding site) and T7 promoter were added to the 5' side, and a gene3 partial sequence serving as a linker for ribosome display was ligated to the 3' side using SfiI.

Acquisition of pH-Dependent Binding scFv from Library by Bead Panning

In order to concentrate only scFv having the ability to bind to SR344, panning was carried out twice by the ribosome display method in accordance with Nature Biotechnology 2000 Dec; 18: 1287-1292. The prepared SR344 was biotinylated using NHS-PEO4-Biotin (Pierce) to obtain an antigen. Panning was carried out using 40 nM of the biotinylated antigen.

Using the resulting DNA pool as a template, HL scFv was restored by PCR using specific primers. After digesting with SfiI, the digested HL scFv was inserted into a phagemide vector pELBG lacI that was also digested with SfiI, and then used to transform XL1-Blue (Stratagene).

Escherichia coli cells carrying the desired plasmid were grown to 0.4 to 0.6 O.D./mL in 2YT medium containing 100 μg/mL ampicillin and 2% glucose. A helper phage (M13KO7, 4.5×10^{11} pfu) was added thereto, statically cultured for 30 minutes at 37°C, and then cultured with shaking for 30 minutes at 37°C. The culture was transferred to a 50 mL Falcon tube, centrifuged for 10 minutes at 3000 rpm, resuspended in 2YT medium containing 100 μg/mL ampicillin, 25 μg/mL kanamycin, and 0.5 mM IPTG, and then incubated overnight at 30°C.

The culture incubated overnight was precipitated with 2.5 M NaCl and 10% PEG, and then diluted with PBS to obtain a phage library solution. 10% M-PBS (PBS containing 10% skim milk) and 1 M Tris-HCl were added to the phage library solution to the final concentration of 2.5% M-PBS and pH 7.4. Panning was carried out by a typical panning method using an

antigen immobilized on 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). More specifically, 40 pmol of biotin-labeled SR344 was added to the prepared phage library and the library was contacted with the antigen for 60 minutes at 37°C. Streptavidin-coated beads (Dynal M-280) washed with 5% M-PBS (PBS containing 5% skim milk) were added and allowed to bind for 15 minutes at 37°C. The beads were washed five times with both 0.5 ml of PBST (PBS containing 0.1% Tween-20, pH 7.4) and PBS (pH 7.4). The beads were then suspended in 1 mL of PBS (pH 5.5) at 37°C, and the phage was recovered immediately. The recovered phage solution was added to 10 mL of logarithmic-growth-phase XL1-Blue (OD600 of 0.4 to 0.5) and allowed to stand for 30 minutes at 37°C for infection. The infected *E. coli* were plated onto a 225 mm x 225 mm plate containing 2 YT, 100 μg/mL ampicillin and 2% glucose. These *E. coli* were used to begin additional phage culture in the same manner as described above and repeat the panning 8 times.

Evaluation by Phage ELISA

The above single colonies were inoculated in 100 μL of 2YT, 100 μg/mL ampicillin, 2% glucose and 12.5 μg/mL tetracycline and cultured overnight at 30°C. 2 μL of this culture was inoculated into 300 μL of 2YT, 100 μg/mL ampicillin and 2% glucose, and then cultured for 4 hours at 37°C. A helper phage (M13KO7) was added to the culture at 9×10^8 pfu, allowed to stand for 30 minutes at 37°C and then shaken for 30 minutes at 37°C for infection. Subsequently, the medium was replaced with 300 μL of 2 YT, 100 μg/mL ampicillin, 25 μg/mL kanamycin, and 0.5 mM IPTG. After culturing overnight at 30°C, the centrifuged supernatant was recovered. 360 μL of 50 mM PBS (pH 7.4) was added to 40 μL of the centrifuged supernatant and subjected to ELISA. A StreptaWell 96-well microtiter plate (Roche) was coated overnight with 100 μL of PBS containing 62.5 ng/mL of biotin-labeled SR344. After removing the antigen by washing with PBST, blocking was carried out with 250 μL of 2% BSA-PBS for 1 hour or more. After removing the 2% BSA-PBS, the prepared culture supernatant was added and allowed to stand for 1 hour at 37°C for antibody binding. After washing, 50 mM PBS (pH 7.4) or 50 mM PBS (pH 5.5) was added and incubated by standing for 30 minutes at 37°C. After washing, detection was carried out with an HRP-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) diluted with 2% BSA-PBS and TMB single solution (Zymed), followed by the addition of sulfuric acid to stop the reaction, and the measurement of absorbance at 450 nm.

However, no clones exhibiting potent pH-dependent binding ability were obtained by this panning using the antigen immobilized on the magnetic beads. Clones that were found to show weak pH-dependent binding ability were subjected to sequence analysis using specific

primers. The positions in these clones where histidine was present at a high rate are shown in Table 3.

[Table 3] Positions of Histidine Substitution Detected Using Phage Library (Magnetic Bead Panning)

H50, H58, H61, H62, H63, H64, H65, H102

L24, L27, L28, L32, L53, L56, L90, L92, L94

Acquisition of pH-Dependently Binding scFv from Library by Column Panning

No clones having strong pH-dependent binding ability were obtained by typical panning using the magnetic bead-immobilized antigen. This may be due to the following reasons. In the panning using an antigen immobilized on magnetic beads or a plate, all phages dissociated from the magnetic beads or plate under acidic conditions are collected. Thus, phage clones having weak pH dependency recovered together reduce the likelihood that clones having strong pH dependency are included in the finally concentrated clones.

Therefore, panning using a column immobilized with an antigen was examined as a more stringent panning method (Fig. 5). There have been no previous reports on the acquisition of clones having pH-dependent binding ability by using panning with an antigen-immobilized column. In the panning using an antigen-immobilized column, when phages that have been bound under neutral conditions are eluted under acidic conditions, clones having weak pH dependency rebind to the antigen within the column and are thereby less eluted, allowing strongly pH-dependent clones that less rebind within the column to be selectively eluted from the column. In addition, although "all" phages that have dissociated under acidic conditions are recovered in the panning using the antigen immobilized on magnetic beads or a plate, the panning using a column immobilized with the antigen enables selective recovery of phages having strong pH-dependent binding ability by allowing an acidic buffer to flow through the column to begin the elution and recovering only "appropriate fractions".

First, a column to which the antigen SR344 was immobilized was prepared. 200 μl of Streptavidin Sepharose (GE Healthcare) was washed with 1 ml of PBS, suspended in 500 μL of PBS, and contacted with 400 pmol of biotin-labeled SR344 for 1 hour at room temperature. Subsequently, an empty column (Amersham Pharmacia Biotech) was filled with the above sepharose and washed with about 3 mL of PBS. The above-mentioned PEG-precipitated library phages were diluted to 1/25 with 0.5% BSA-PBS (pH 7.4), passed through a 0.45 nm filter, and then added to the column. After washing with about 6 mL of PBS (pH 7.4), 50 mM MES-NaCl (pH5.5) was allowed to flow through the column to elute antibodies that dissociate under low pH. The appropriate eluted fractions were collected, and the recovered phage

solution was added to 10 mL of logarithmic-growth-phase XL1-Blue (OD600 of 0.4 to 0.5) and allowed to stand for 30 minutes at 37°C.

The infected *E. coli* were plated onto a 225 mm x 225 mm plate containing 2YT, 100 μg/mL ampicillin, and 2% glucose. These *E. coli* were used to begin additional phage culture in the same manner as described above and repeat the panning 6 times.

Evaluation by Phage ELISA

The resulting phages were evaluated by phage ELISA. Clones that were found to have strong pH dependency were subjected to sequence analysis using specific primers. As a result, several clones showing strong pH-dependent binding as compared to WT were obtained. As shown in Fig. 6, clone CL5 (H chain: CLH5, L chain: CLL5) (CLH5: amino acid sequence of SEQ ID NO: 5, CLL5: amino acid sequence of SEQ ID NO: 8) was found to exhibit particularly strong pH-dependent binding as compared to WT. It was thus confirmed that antibodies exhibiting strong pH-dependent binding, while being unable to be obtained by typical panning using the antigen immobilized onto magnetic beads, can be obtained by panning using a column immobilized with the antigen. Therefore, panning using an antigen-immobilized column was found to be a highly effective method for obtaining pH-dependently binding antibodies from a library. The amino acid sequences of the clones showing pH-dependent binding were analyzed, and the positions where histidine was present at a high probability in the concentrated clones are shown in Table 4.

[Table 4] Positions of Histidine Substitution found by Phage Library (Column Panning)
H31, H50, H58, H62, H63, H65, H100b, H102
L24, L27, L28, L32, L53, L56, L90, L92, L94

[Example 4] Expression and Purification of Histidine-Modified Humanized IL-6 Receptor Antibody

Production, Expression and Purification of Expression Vector of Histidine-Modified Humanized IL-6 Receptor Antibody

In order to convert clones showing strong pH dependency in phage ELISA to IgG, VH and VL were respectively amplified by PCR, digested with XhoI/NheI and EcoRI, and inserted to a mammalian cell expression vector. The nucleotide sequence of each DNA fragment was determined by a method known to persons skilled in the art. CLH5/L73, in which CLH5 was used for the H chain and L73 obtained in Example 2 was used for the L chain, was expressed and purified as IgG. Expression and purification were carried out by the method described in Example 1.

Antibody having even higher pH dependency was produced by combining mutation sites. Based on the locations where His was concentrated in the phage library as well as the structural information and the like, H32, H58, H62 and H102 in H3pI which was obtained as an H chain in Example 2 were substituted with histidine, and H95 and H99 were further substituted with valine and isoleucine, respectively, to produce H170 (SEQ ID NO: 4). The variant production was carried out using the method described in Example 1. In addition, L82 (SEQ ID NO: 7) was produced by substituting the 28th histidine of L73, which was produced as an L chain in Example 2, with aspartic acid. The variant production was carried out using the method described in Example 1. H170/L82, in which H170 was used for the H chain and L82 was used for the L chain, was expressed and purified as IgG using the method described in Example 1.

[Example 5] Evaluation of IL-6R-Neutralizing Activity of pH-Dependent Binding Antibody

Evaluation of Human IL-6 Receptor-Neutralizing Activity of Clones Converted to IgG

The IL-6 receptor-neutralizing activity was evaluated for four antibodies: humanized PM1 antibody (WT) and H3pI/L73, CLH5/L73 and H170/L82 produced in Examples 2 and 4.

More specifically, the IL-6 receptor-neutralizing activity was evaluated using BaF3/gp130 exhibiting IL-6/IL-6 receptor-dependent growth. BaF3/gp130 was washed three times with RPMI1640 medium containing 10% FBS, then suspended at 5 x 10^4 cells/mL in RPMI1640 medium containing 60 ng/mL of human interleukin-6 (Toray), 60 ng/mL of recombinant soluble human IL-6 receptor (SR344) and 10% FBS. 50 μL of the suspension was dispensed into each of the wells of a 96-well plate (Corning). Next, the purified antibody was diluted with RMPI1640 containing 10% FBS, and 50 μl of the antibody was mixed into each well. After culturing for 3 days at 37°C and 5% CO_2, WST-8 reagent (Cell Counting Kit-8, Dojindo Laboratories) diluted two-fold with PBS was added at 20 μL/well, and then immediately measured for absorbance at 450 nm (reference wavelength: 620 nm) using the Sunrise Classic (Tecan). After culturing for 2 hours, absorbance at 450 nm was measured again (reference wavelength: 620 nm). The IL-6 receptor-neutralizing activity was evaluated based on the change in absorbance after 2 hours. As a result, as shown in Fig. 7, H3pI/L73, CLH5/L73 and H170/L82 were shown to have equivalent biological neutralization activity in comparison with the humanized PM1 antibody (WT).

[Example 6] Biacore Analysis of pH-Dependently Binding Antibody

Analysis of Binding of pH-Dependently Binding Clones to Soluble IL-6 Receptor

Kinetic analyses of antigen-antibody reactions at pH 5.8 and pH 7.4 were carried out using Biacore T100 (GE Healthcare) on the four antibodies: humanized PM1 antibody (WT) and

H3pI/L73, CLH5/L73, and H170/L82 produced in Examples 2 and 4 (buffer: 10 mM MES (pH 7.4 or pH 5.8), 150 mM NaCl, and 0.05% Tween 20). Various antibodies were bound onto a sensor chip immobilized with recomb-protein A/G (Pierce) by amine coupling. SR344 adjusted to concentrations of 9.8 to 400 nM was injected to the chip as an analyte. Association and dissociation of the pH-dependent binding clones to SR344 was observed in real time (Figs. 8 and 9). All the measurements were carried out at 37°C. Association rate constants k_a (1/Ms) and dissociation rate constants k_d (1/s) were calculated using Biacore T100 Evaluation Software (GE Healthcare), and dissociation constants KD (M) were calculated on the basis of those values (Table 5). Moreover, the ratio of the affinities at pH 5.8 and pH 7.4 were calculated for each clone to evaluate pH-dependent binding. All the measurements were carried out at 37°C.

As a result of calculating the affinity ratio between pH 5.8 and pH 7.4 for each clone, the pH-dependent binding (affinity) of H3pI/L73, H170/L82 and CLH5/L73 to SR344 was 41-fold, 394-fold and 66-fold, respectively, each showing the pH-dependent binding more than 15 times higher than WT.

Anti-IL-6 receptor antibodies that strongly bind to the antigen at the plasma pH of 7.4 but weakly bind to the antigen at the intraendosomal pH of 5.5 to 6.0 have not been reported yet. In this study, antibodies were obtained that retain the biological neutralization activity equivalent to the WT humanized IL-6 receptor antibody and the affinity at pH 7.4, but exhibit the affinity at pH 5.8 that has been specifically lowered more than 10 times.

[Table 5] Comparison of Binding of pH-Dependently Binding Clones Directed Against SR344 to Soluble IL-6 Receptor

	pH7.4			pH5.8			
	ka(1/Ms)	kd(1/s)	KD(M)	ka(1/Ms)	kd(1/s)	KD(M)	KD(pH5.8)/KD(pH7.4)
WT	5.1E+05	1.0E-03	2.1E-09	7.6E+05	3.8E-03	5.0E-09	**2.4**
H3pI/L73	5.4E+05	7.4E-04	1.4E-09	1.7E+05	9.7E-03	5.7E-08	**41.3**
H170/L82	6.8E+05	1.1E-03	1.6E-09	2.6E+04	1.7E-02	6.4E-07	**393.5**
CLH5/L73	7.1E+05	7.9E-04	1.1E-09	3.8E+05	2.8E-02	7.4E-08	**66.1**

Analysis of Binding of pH-Dependently Binding Clones to Membrane IL-6 Receptor

Antigen-antibody reactions to membrane IL-6 receptor at pH 5.8 and pH 7.4 were observed for the above produced pH-dependent binding clones, using Biacore T100 (GE Healthcare). The binding to membrane IL-6 receptor was evaluated by evaluating the binding to the IL-6 receptor immobilized onto a sensor chip. SR344 was biotinylated according to a method known to persons skilled in the art, and the biotinylated SR344 was immobilized on the sensor chip via streptavidin by utilizing the affinity between streptavidin and biotin. All the measurements were carried out at 37°C, and the mobile phase buffer contained 10 mM MES (pH

5.8), 150 mM NaCl and 0.05% Tween 20. The pH-dependent binding clones were injected therein under the condition of pH 7.4 and allowed to bind to SR344 (injection sample buffer: 10 mM MES (pH 7.4), 150 mM NaCl, 0.05% Tween 20), and the pH-dependent dissociation of each clone at the mobile phase pH of 5.8 was observed (Fig. 10).

The dissociation rate (k_d(1/s)) at pH 5.8 was calculated using Biacore T100 Evaluation Software (GE Healthcare) by fitting only the dissociation phase at pH 5.8, where 0.5 μg/mL of the sample was bound in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, and dissociated in 10 mM MES (pH 5.8), 150 mM NaCl, and 0.05% Tween 20. Similarly, the dissociation rate (k_d(1/s)) at pH 7.4 was calculated using Biacore T100 Evaluation Software (GE Healthcare) by fitting only the dissociation phase at pH 7.4, where 0.5 μg/mL of the sample was bound in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, and dissociated in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20. The pH-dependent dissociation rate constant of each clone is shown in Table 6.

[Table 6] Comparison of Rate Constant of Dissociation of pH-Dependent Binding Clones directed against SR344 from Membrane IL-6 Receptor

	kd(1/s)		kd ratio
	pH7.4	pH5.8	pH5.8/pH7.4
WT	4.84E-04	7.15E-04	1.5
H3pI/L73	3.44E-04	3.78E-03	11.0
H170/L82	7.70E-04	1.44E-03	1.9
CLH5/L73	1.04E-03	5.67E-03	5.5

The highest pH dependency of the dissociation ratio was observed in H3pI/L73 followed by CLH5/L73 and H170/L82 in descending order, and each clone demonstrated higher pH-dependent dissociation from the membrane IL-6 receptor than WT. However, the rank of pH-dependent association/dissociation was different between the soluble IL-6 receptor and membrane IL-6 receptor. It was revealed that H170/L82, which exhibited the highest pH-dependent binding in the analysis of binding to soluble IL-6 receptor, showed the lowest pH-dependent binding in the analysis of binding to the membrane IL-6 receptor. In general, it is known that while IgG molecules monovalently bind to a soluble antigen (affinity), they divalently bind to membrane antigens (avidity). It is suggested that this difference in the binding mode between soluble antigens and membrane antigens influenced the pH-dependent binding of H170/L82.

[Example 7] Confirmation of Multiple Binding to Antigen by pH-Dependent Binding Antibody

As described in Example 2, pH-dependent binding antibodies may be able to bind to antigens multiple times. Specifically, a pH-dependent binding antibody that has bound to an antigen is non-specifically taken up into endosomes, but dissociated from the soluble antigen under the intraendosomal acidic conditions. The antibody binds to FcRn and thereby returns to the plasma. Since the antibody that has returned to the plasma is not bound to antigen, it is able to bind to a new antigen again. The repetition of this process enables pH-dependent binding antibodies to bind to antigens multiple times. However, for IgG antibodies that do not have pH-dependent binding ability, not all antigens are dissociated from the antibodies under the intraendosomal acidic conditions. Thus, such antibodies that have been returned to the plasma by FcRn remain bound to antigen, and therefore cannot bind to new antigens. Consequently, in nearly all cases, each single molecule of IgG antibodies is able to neutralize only two antigens (in the case of divalent binding).

Therefore, it was evaluated whether the three pH-dependently binding antibodies (H3pI/L73, CLH5/L73, and H170/L82) constructed in Examples 2 and 4 were able to bind to the antigen SR344 multiple times as compared to the humanized PM1 antibody (wild type, WT).

Biacore (GE Healthcare) was used to evaluate that the antibodies binding at pH 7.4 and dissociating at pH 5.8 were able to bind to the antigen multiple times. The antibody to be evaluated was bound to a recomb-protein A/G (Pierce)-immobilized sensor chip by the amine coupling method, and a mobile phase of pH 7.4 was allowed to flow (step 1). SR344 solution adjusted to pH 7.4 was then allowed to flow as an analyte to bind SR344 to the antibody at pH 7.4 (step 2). This binding at pH 7.4 mimics the antigen binding in plasma. Subsequently, buffer adjusted to pH 5.8 alone (not containing SR344) was added as an analyte to expose the antigen bound to the antibody to acidic conditions (step 3). This dissociation at pH 5.8 mimics the binding state of antigen-antibody complexes in endosomes. Subsequently, step 2 was repeated. This mimics the rebinding of antibody that has been returned to plasma by FcRn to a new antigen. Subsequently, step 2 was repeated to expose the antibody-antigen complex to acidic conditions. Repeating "step 2 to step 3" multiple times at 37°C as described above can mimic the *in vivo* state in which antibodies are repeatedly taken up from the plasma into endosomes by pinocytosis and returned to the plasma by FcRn (Nat. Rev. Immunol. 2007 Sep; 7(9): 715-25).

The produced pH-dependent binding clones described above were analyzed using Biacore T100 (GE Healthcare) for their ability to bind to the antigen SR344 multipe times at pH 5.8 and pH 7.4. More specifically, the analysis was carried out as follows. All the measurements were carried out at 37°C. First, the sample antibodies described above were bound onto a recomb-protein A/G (Pierce)-immobilized sensor chip by amine-coupling, where the mobile phase buffer was 10 mM MES (pH 5.8), 150 mM NaCl, and 0.05% Tween 20 (step 1).

SR344 adjusted to a concentration of about 40 nM was injected as an analyte for 3 minutes at pH 7.4 and allowed to bind (the buffer for injected SR344 was 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20) (step 2). Subsequently, the injection of SR344 was discontinued and a mobile phase of pH 5.8 was allowed to flow for about 70 seconds to expose the antibody/SR344 complex under acidic conditions (step 3). Ten sets of this binding (step 2)/acidity exposure (step 3) process were continuously repeated to observe the sensorgram in real-time, which is shown in Fig. 11. WT showed less dissociation of SR344 during the acidic exposure in step 3, and consequently the proportion of antibody capable of binding to new antigens in the subsequent step 2 was extremely low. In contrast, it was found that the pH-dependent binding clones, particularly H170/L82 and CLH5/L73, demonstrated so strong dissociation during the acidic exposure in step 3 that most of the bound SR344 was dissociated, and therefore nearly all antibodies were able to bind to new antigens in the subsequent step 2. In the 10-set repetition of the binding (step 2) and acidic exposure (step 3), almost all H170/L82 and CLH5/L73 antibodies were able to bind to new antigens in each set.

The obtained sensorgrams were used to calculate the binding amount of SR344 in each set for each sample using Biacore T100 Evaluation Software (GE Healthcare). The integrated values in the time course of the 10 sets are shown in Fig. 12. The integrated RU values obtained at the 10th set are equivalent to the total amount of antigens bound during the ten cycles. The pH-dependent binding clones, particularly H170/L82 and CLH5/L73, showed the largest total amounts of bound antigens in comparison with WT, and were demonstrated to be able to repeatedly bind to roughly four times the amount of antigens bound by WT. Accordingly, it was revealed that by conferring pH-dependent binding ability to WT, such antibodies can repeatedly bind to antigens and thereby neutralize multiple antigens.

[Example 8] PK/PD Test of pH-Dependently-Binding Antibody Using Human IL-6 Receptor Transgenic Mice

IL-6 receptors are present in the body in both soluble IL-6 receptor form and membrane-type IL-6 receptor form (Nat. Clin. Pract. Rheumatol. 2006 Nov; 2(11): 619-26). Anti-IL-6 receptor antibodies bind to soluble IL-6 receptors and membrane-type IL-6 receptors and neutralize their biological action. It is believed that an anti-IL-6 receptor antibody binds to a membrane-type IL-6 receptor, is subsequently taken up into an endosome within a cell by internalization while the antibody is kept bound to the membrane-type IL-6 receptor, and then moves to a lysosome while still kept bound to the membrane-type IL-6 receptor where it is degraded by lysosome together with the membrane-type IL-6 receptor. If H3pI/L73, CLH5/L73, and H170/L82, which are the pH-dependent-binding IL-6 receptor antibodies evaluated in Example 6, are able to return to plasma via FcRn as a result of dissociation under

acidic conditions within endosomes, the antibodies that have returned to the plasma can bind to antigens again. This enables neutralization of multiple membrane-type IL-6 receptors with a single antibody molecule. Whether or not the return to the plasma via FcRn as a result of dissociation under acidic conditions within endosomes is achieved with the constructed pH-dependent-binding anti-IL-6 receptor antibodies can be determined by evaluating whether the pharmacokinetics of these antibodies are improved as compared with that of WT.

Thus, the pharmacokinetics in human-IL-6-receptor transgenic mice (hIL-6R tg mice, Proc. Natl. Acad. Sci. USA 1995 May 23; 92(11): 4862-6) was evaluated for the four types of antibodies, that is, humanized PM1 antibody (wild type: WT) and H3pI/L73, CLH5/L73, and H170/L82 constructed in Examples 2 and 4. WT, H3pI/L73, CLH5/L73, or H170/L82 was administered by single-dose intravenous administration to hIL-6R tg mice at 25 mg/kg, and blood samples were collected, before administration and over time. Collected blood was immediately centrifuged for 15 minutes at 15,000 rpm and 4°C to obtain plasma. Separated plasma was stored in a freezer set to -20°C or lower until measurements were carried out.

The measurement of concentration in mouse plasma was carried out by ELISA. Samples for calibration curve were prepared at plasma concentrations of 6.4, 3.2, 1.6, 0.8, 0.4, 0.2 and 0.1 μg/mL. The calibration curve samples and mouse plasma measurement samples were dispensed into an immunoplate (Nunc-Immuno Plate, MaxiSorp (Nalge Nunc International)) immobilized with anti-human IgG (γ-chain specific) F(ab')2 (Sigma), and allowed to stand undisturbed for one hour at room temperature. Goat anti-human IgG-BIOT (Southern Biotechnology Associates) and streptavidin-alkaline phosphatase conjugate (Roche Diagnostics) were sequentially allowed to react, and a chromogenic reaction was carried out by using BluePhos Microwell Phosphatase Substrates System (Kirkegaard & Perry Laboratories) as substrate. Absorbance at 650 nm was measured with a microplate reader. The concentrations in mouse plasma were calculated from the absorbance of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices). Time courses of plasma concentrations of WT as well as H3pI/L73, CLH5/L73, and H170/L82 are shown in FIG. 13.

The pharmacokinetics was improved for all of H3pI/L73, CLH5/L73, and H170/L82 as compared with WT. In particular, the pharmacokinetics of H3pI/L73 and CLH5/L73 were improved considerably. A wild type anti-IL-6 receptor antibody (WT) bound to membrane-type IL-6 receptor is taken up into an endosome within a cell by internalization, moves to a lysosome while the antibody is kept bound to the antigen, and then degraded; therefore, it has a short residence time in the plasma. In contrast, since the pharmacokinetics of the pH-dependent-binding anti-IL-6 receptor antibodies were improved considerably, the pH-dependent-binding anti-IL-6 receptor antibodies were thought to return to the plasma again

via FcRn as a result of dissociation from the antigen, membrane-type IL-6 receptor, under acidic conditions within endosomes.

Although the pharmacokinetics was improved for all of H3pI/L73, CLH5/L73, and H170/L82 as compared with WT, the effect of prolonging plasma persistence time of H170/L82 was weaker than that of H3pI/L73 and CLH5/L73. Since IgG molecules are thought to normally bind divalently to membrane-bound antigen, it is thought that anti-IL-6 receptor antibodies also bind divalently (avidity) to membrane-type IL-6 receptors and then are internalized. As indicated in Example 6, the analysis using Biacore revealed that H170/L82 rapidly dissociated from the IL-6 receptor at pH 5.8 when binding to soluble IL-6 receptor (FIG. 9), but the dissociation rate thereof from the IL-6 receptor at pH 5.8 when binding to membrane-type IL-6 receptor was extremely slow (FIG. 10). From this result, the reason for the weak effect of prolonging residence time in plasma of H170/L82 is thought to be that the antibody was unable to adequately dissociate within endosomes after having been internalized due to its slow dissociation at pH 5.8 when binding to membrane-type IL-6 receptor. Namely, as for the case relating to membrane antigens, it was determined that in order for a single IgG molecule to neutralize multiple membrane antigens, the pH dependency of dissociation from divalent binding (avidity) is more important than the pH dependency of monovalent binding (affinity).

[Example 9] PK/PD Test of pH-Dependently-Binding Antibody Using Cynomolgus Monkeys

Since the pharmacokinetics of the pH-dependently-binding anti-IL-6 receptor antibodies were improved considerably in Example 8, the pH-dependently-binding anti-IL-6 receptor antibodies were thought to return to plasma via FcRn as a result of dissociation from the antigen, membrane-type IL-6 receptor, under acidic conditions within endosomes. If antibodies that have returned to the plasma can bind to membrane-type IL-6 receptors again, neutralization of an antigen, the membrane-type IL-6 receptor, by pH-dependently-binding anti-IL-6 receptor antibodies is thought to persist longer than that by the wild-type anti-IL-6 receptor antibody, at the same dosage. In addition, since the soluble IL-6 receptor is also present among IL-6 receptors, the duration of neutralization is thought to be longer for the same dosage with respect to the soluble IL-6 receptor as well.

The pharmacokinetics in cynomolgus monkeys was evaluated for WT and H3pI/L73. WT or H3pI/L73 was administered to cynomolgus monkeys by single-dose intravenous administration at 1 mg/kg, and blood samples were collected, before administration and over time. Collected blood was immediately centrifuged for 15 minutes at 15,000 rpm and 4°C to obtain plasma. The separated plasma was stored in a freezer set to -20°C or lower until measurements were carried out.

The measurement of concentration in cynomolgus monkey plasma was carried out by ELISA. First, anti-human IgG (γ-chain specific) F(ab')2 fragment of antibody (Sigma) was dispensed into a Nunc-ImmunoPlate MaxiSorp (Nalge Nunc International) and allowed to stand undisturbed overnight at 4°C to prepare plates immobilized with anti-human IgG. Calibration curve samples having plasma concentrations of 3.2, 1.6, 0.8, 0.4, 0.2, 0.1 and 0.05 μg/mL and cynomolgus monkey plasma measurement samples diluted 100-fold or more were prepared; 200 μL of 20 ng/mL cynomolgus monkey IL-6R was added to 100 μL of the calibration curve samples and plasma measurement samples; and then, they were allowed to stand undisturbed for one hour at room temperature. Subsequently, the samples were dispensed into the anti-human IgG-immobilized plate and allowed to stand undisturbed for another one hour at room temperature. Biotinylated Anti-Human IL-6R Antibody (R&D) was allowed to react for one hour at room temperature, and then Streptavidin-PolyHRP80 (Stereospecific Detection Technologies) was allowed to react for one hour. A chromogenic reaction was carried out by using TMP One Component HRP Microwell Substrate (BioFX Laboratories) as substrate, then, the reaction was stopped with 1N sulfuric acid (Showa Chemical) and the absorbance at 450 nm was measured with a microplate reader. The concentrations in cynomolgus monkey plasma were calculated from the absorbance of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices). The time courses of plasma concentrations of WT and H3pI/L73 after the intravenous administration are shown in FIG. 14. As a result, the pharmacokinetics of H3pI/L73 was improved considerably in comparison with WT in cynomolgus monkeys in the same manner as in human IL-6 receptor transgenic mice. Since the pharmacokinetics of a pH-dependent-binding anti-IL-6 receptor antibody, H3pI/L73, was improved considerably, H3pI/L73 was thought to return to the plasma via FcRn as a result of dissociation from the antigen, membrane-type IL-6 receptor, under acidic conditions within endosomes.

In order to evaluate the degree to which cynomolgus monkey membrane-type IL-6 receptor is neutralized by the intravenous administration of WT and H3pI/L73, the effects of sample antibodies on plasma C-reactive protein (CRP) induced by cynomolgus monkey IL-6 were studied. Since CRP is secreted when IL-6 binds to membrane-type IL-6 receptors, CRP serves as an indicator of neutralization of membrane-type IL-6 receptors. Cynomolgus monkey IL-6 (cyno.IL-6 prepared in Example 1) containing 1% inactivated cynomolgus monkey plasma was administered subcutaneously into lower backs of the animals daily at 5 μg/kg from day 3 to day 10 after the administration of WT or H3pI/L73. Blood samples were collected from the saphenous vein immediately before the start of cynomolgus monkey IL-6 administration (day 3) and after the administration at 24-hour intervals (day 4 to day 11), then were separated into plasma. The CRP concentrations of individual animals were measured with Cias R CRP

(Kanto Chemical) using an automated analyzer (TBA-120FR, Toshiba Medical Systems). The time courses of CRP concentration upon induction with cynomolgus IL-6 with respect to WT and H3pI/L73 are shown in FIG. 15. As a result, the duration of CRP suppression was prolonged considerably by H3pI/L73 in comparison with WT. On the basis of this finding, a pH-dependent-binding anti-IL-6 receptor antibody, H3pI/L73, was thought to return to the plasma via FcRn as a result of dissociation from its antigen, membrane-type IL-6 receptor, under acidic conditions within endosomes; and neutralize the membrane-type IL-6 receptor by re-binding thereto; and thereby suppress production of CRP for a longer period of time than WT. In other words, H3pI/L73 was shown to be able to bind to and neutralize the membrane-type IL-6 receptor more than once, as a single antibody molecule. Since the duration of suppression of CRP production by H3pI/L73 is prolonged in comparison to that by WT, the duration of time where an antigen, the membrane-type IL-6 receptor, is bound by antibodies was indicated to be prolonged for H3pI/L73 than WT.

In order to evaluate the degree to which cynomolgus monkey soluble IL-6 receptor is neutralized by the intravenous administration of WT and H3pI/L73, the concentration of unbound cynomolgus monkey soluble IL-6 receptor in cynomolgus monkey plasma was measured. All IgG-type antibodies (cynomolgus monkey IgG, anti-human IL-6 receptor antibody, and a complex of anti-human-IL-6 receptor antibody and cynomolgus monkey soluble IL-6 receptor) present in the plasma were adsorbed to Protein A by adding 30 μL of cynomolgus monkey plasma to an appropriate amount of rProtein A Sepharose Fast Flow (GE Healthcare) resin dried in a 0.22 μm filter cup (Millipore). After spinning down with a high-speed centrifuge, the solution that passed through (hereinafter referred to as "pass solution") was recovered. Since the pass solution does not contain the complex of anti-human IL-6 receptor antibody and cynomolgus monkey soluble IL-6 receptor which is bound to protein A, the concentration of unbound soluble IL-6 receptor can be measured by measuring the concentration of cynomolgus monkey soluble IL-6 receptor in the pass solution. Monoclonal Anti-human IL-6R Antibody (R&D) that was ruthenium-labeled with Sulfo-Tag NHS Ester (Meso Scale Discovery) and Biotinylated Anti-human IL-6R Antibody (R&D) were mixed with cynomolgus monkey IL-6 receptor calibration curve samples adjusted to concentrations of 4000, 2000, 1000, 500, 250, 125, and 62.5 pg/mL and the plasma samples treated with Protein A as described above. The mixtures were allowed to react for one hour at room temperature. Subsequently, the mixtures were dispensed into an SA-Coated Standard MA2400 96-well plate (Meso Scale Discovery). After allowing to react for another one hour and washing, Read Buffer T (x4) (Meso Scale Discovery) was dispensed. Immediately thereafter, the measurement with Sector Imager 2400 (Meso Scale Discovery) was conducted. The concentrations of cynomolgus monkey IL-6 receptor were calculated from the response of the calibration curve by using the

analytical software, SOFTmax PRO (Molecular Devices). The time courses of concentrations of unbound cynomolgus monkey soluble IL-6 receptor for WT and H3pI/L73 are shown in FIG. 16. As a result, the duration of neutralization of cynomolgus monkey soluble IL-6 receptor by H3pI/L73 was considerably prolonged as compared to that by WT. On the basis of this finding, the pH-dependent-binding anti-IL-6 receptor antibody H3pI/L73 was thought to dissociate from its antigen, soluble IL-6 receptor, under acidic conditions in endosomes; and return to the plasma via FcRn; and bind to and neutralize the soluble IL-6 receptor again. Since the duration of suppression of unbound cynomolgus monkey soluble IL-6 receptor by H3pI/L73 is prolonged in comparison to that by WT, the duration of time where an antigen, the soluble IL-6 receptor, is bound by antibodies was indicated to be prolonged for H3pI/L73 than WT.

From these findings, the time until the antibody disappears from the plasma as well as the time where soluble and membrane-type IL-6 receptors are bound by the antibody in the body were found to be considerably elongated for the pH-dependent-binding anti-IL-6 receptor antibodies that were made to bind strongly to the antigen at pH 7.4, which is the pH in the plasma, but bind weakly to the antigen at pH 5.8, which is the pH within endosomes, as compared to the wild-type anti-IL-6 receptor antibody. This makes it possible to reduce the dosage and frequency of administration to patients, and in consequence, the total administration dosage. Therefore, the pH-dependent-binding anti-IL-6 receptor antibody is thought to be particularly advantageous as a pharmaceutical for use as an IL-6 antagonist.

[Example 10] Improvement of pH-Dependent Binding to Membrane-Type IL-6 Receptor by Optimization of Variable Region

Optimization of Variable Regions H3pI/L73 and CLH5/L82

Antibodies having pH-dependent binding abilities were shown to demonstrate superior effects in Example 9. Therefore, to further improve the pH-dependent binding abilities, mutations were introduced into the CDR sequence of CLH5 obtained in Example 3 to construct VH1-IgG1 (SEQ ID NO: 21) and VH2-IgG1 (SEQ ID NO: 22). In addition, mutations were introduced into the framework sequence and CDR sequence of H3pI to construct the modified H chains VH3-IgG1 (SEQ ID NO: 23) and VH4-IgG1 (SEQ ID NO: 24). Mutations were introduced into the CDR sequences of L73 and L82 to construct the modified L chains VL1-CK (SEQ ID NO: 25), VL2-CK (SEQ ID NO: 26), and VL3-CK (SEQ ID NO: 27). More specifically, the mutants were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the method described in the appended instructions, and the resulting plasmid fragments were inserted into an mammalian cell expression vector to construct the desired H chain expression vectors and L chain expression vectors. The nucleotide sequences

of the resulting expression vectors were determined by methods known to persons with ordinary skill in the art.

The antibody having VH2-IgG1 (SEQ ID NO: 22) as H chain and VL2-CK (SEQ ID NO: 26) as L chain was denoted as Fv1-IgG1, the antibody having VH1-IgG1 (SEQ ID NO: 21) as H chain and L82 as L chain was denoted as Fv2-IgG1, the antibody having VH4-IgG1 (SEQ ID NO: 24) as H chain and VL1-CK (SEQ ID NO: 25) as L chain was denoted as Fv3-IgG1, and the antibody having VH3-IgG1 (SEQ ID NO: 23) as H chain and VL3-CK (SEQ ID NO: 27) as L chain was denoted as Fv4-IgG1. Of these, Fv2-IgG1 and Fv4-IgG1 were expressed and purified. The expression and purification were carried out by the method described in Example 1.

Analysis of Binding of pH-Dependent-Binding Clones to Soluble IL-6 Receptor

Kinetic analyses of antigen-antibody reactions at pH 7.4 were carried out on the four types of antibodies, i.e., the humanized PM1 antibody (wild type: WT), and WT, H3pI/L73-IgG1, Fv2-IgG1, and Fv4-IgG1 constructed in Examples 2 and 10, by using Biacore T100 (GE Healthcare) (buffer: 10 mM MES (pH 7.4), 150 mM NaCl, 0.05% Tween 20). Each antibody was bound on a sensor chip on which an anti-IgG γ chain specific $F(ab)_2$ (Pierce) was immobilized by amine coupling, and then, SR344 adjusted to a concentration of 9.8 to 40 nM was injected thereto as an analyte. The association to and dissociation from SR344 were observed on a real-time basis for the pH-dependent-binding clones. All the measurements were carried out at 37°C. Association rate constants k_a (1/Ms) and dissociation rate constants k_d (1/s) were calculated using Biacore T100 Evaluation Software (GE Healthcare), and dissociation constants KD (M) were calculated on the basis of those values (Table 7).

[Table 7] Comparison of Dissociation Rate Constants of pH-Dependent-Binding Clones from Soluble IL-6 Receptor, SR344

Sample	k_a (1/Ms)	k_d (1/s)	KD (M)
WT	4.0E+05	1.1E-03	2.7E-09
H3pI/L73	4.1E+05	5.9E-04	1.4E-09
Fv2-IgG1	3.9E+05	7.7E-04	2.0E-09
Fv4-IgG1	7.2E+05	1.0E-03	1.4E-09

As a result of calculating the affinity at pH 7.4 for each clone, the dissociation constants (affinity, KD value) of WT, H3pI/L73-IgG1, Fv2-IgG1, and Fv4-IgG1 to SR344 were, respectively, 2.7 nM, 1.4 nM, 2.0 nM, and 1.4 nM, and they are nearly equivalent. Fv2-IgG1

and Fv4-IgG1 were demonstrated to have binding ability to the soluble IL-6 receptor that is equal to or greater than that of WT.

Analysis of Binding of pH-Dependent-Binding Clones to Membrane-Type IL-6 Receptor

Antigen-antibody reactions to membrane-type IL-6 receptor were observed at pH 5.8 and pH 7.4 for the four types of constructed clones, WT, H3pI/L73-IgG1, Fv2-IgG1, and Fv4-IgG1 by using Biacore T100 (GE Healthcare). Binding to the membrane-type IL-6 receptor was evaluated by evaluating binding to the IL-6 receptor immobilized on a sensor chip. SR344 was biotinylated in accordance with a method known among persons with ordinary skill in the art, and the biotinylated SR344 was immobilized on the sensor chip via streptavidin using the affinity between streptavidin and biotin. All the measurements were carried out at 37°C. The mobile phase buffer was 10 mM MES (pH 5.8), 150 mM NaCl and 0.05% Tween 20. The pH-dependent-binding clones were injected therein under conditions of pH 7.4 to allow them to bind to SR344 (injection sample buffer: 10 mM MES (pH 7.4), 150 mM NaCl, 0.05% Tween 20), then, pH-dependent dissociation of each clone was observed at the pH of the mobile phase of 5.8 (FIG. 17).

Sample concentrations were adjusted to 0.25 μg/mL. Binding was carried out in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20. Dissociation was carried out in 10 mM MES (pH 5.8), 150 mM NaCl, and 0.05% Tween 20. For this case, the dissociation rate constants (k_d(1/s)) at pH 5.8 were calculated by fitting only the dissociation phase at pH 5.8 using Biacore T100 Evaluation Software (GE Healthcare). In a similar manner, sample concentrations were adjusted to 0.25 μg/mL, binding was carried out in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, dissociation was carried out in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, and the dissociation rate constants (k_d(1/s)) at pH 7.4 were calculated by fitting only the dissociation phase at pH 7.4 using Biacore T100 Evaluation Software (GE Healthcare). The pH-dependent dissociation rate constants of each clone are shown in Table 8.

[Table 8] Comparison of Dissociation Rate Constants of pH-Dependent-Binding Clones from Membrane-Type IL-6 Receptor, SR344

Sample	pH7.4 kd (1/s)	pH5.8 kd (1/s)	pH Dependency kd(pH5.8)/kd(pH7.4)
WT	2.5E-04	2.5E-04	1.00
H3pI/L73	2.6E-04	6.7E-04	2.59
Fv2-IgG1	3.4E-04	2.4E-03	7.18
Fv4-IgG1	4.7E-04	2.6E-03	5.56

As a result of calculating pH dependency for each clone, the pH dependencies of binding to the membrane-type IL-6 receptor of the four clones, WT, H3pI/L73-IgG1, Fv2-IgG1, and Fv4-IgG1 with respect to SR344 were 1.0-fold, 2.59-fold, 7.18-fold, and 5.56-fold, respectively. Fv2-IgG1 and Fv4-IgG1 demonstrated higher pH-dependency in dissociation from the membrane-type IL-6 receptor than H3pI/L73-IgG1.

On the basis of the above, Fv2-IgG1 and Fv4-IgG1 were shown to demonstrate stronger pH-dependent binding to the membrane-type IL-6 receptor than H3pI/L73-IgG1 while maintaining the affinity for the soluble IL-6 receptor equal to or stronger than that of WT.

[Example 11] PK/PD Test of pH-Dependent-Binding Antibodies with Optimized Variable Regions Using Human IL-6 Receptor Transgenic Mice

The pharmacokinetics of Fv2-IgG1 and Fv4-IgG1, as well as WT and H3pI/L73-IgG1 prepared and evaluated in Example 10 were evaluated using the human IL-6 receptor transgenic mice used in Example 8. WT, H3pI/L73-IgG1, Fv2-IgG1, or Fv4-IgG1 was administered by single-dose intravenous administration to hIL-6R tg mice at 25 mg/kg, and the concentration of each antibody in the plasma was measured in the same manner as in Example 8. The time courses of the plasma concentrations of WT, H3pI/L73-IgG1, Fv2-IgG1, and Fv4-IgG1 are shown in FIG. 18.

The pharmacokinetics of H3pI/L73-IgG1 improved in comparison with WT in the same manner as in Example 8, while the pharmacokinetics of Fv2-IgG1 and Fv4-IgG1 was further improved than H3pI/L73-IgG1. Measurement as to the unbound IL-6 receptor concentrations, as measured in cynomolgus monkeys in Example 9, was carried out in the hIL-6R tg mice in this test using the same method. As a result, prolongation of the duration of neutralization of the soluble IL-6 receptor was confirmed for Fv2-IgG1 and Fv4-IgG1 in comparison to that for H3pI/L73-IgG1 (data not shown). As indicated in Example 10, the pH-dependent binding to the membrane-type IL-6 receptor was improved for Fv2-IgG1 and Fv4-IgG1 as compared with H3pI/L73-IgG1. Therefore, it was indicated that further improvement in the pharmacokinetics and duration of neutralization of the soluble IL-6 receptor over those of H3pI/L73-IgG1 is possible by improving the pH-dependent binding to the membrane-type IL-6 receptor.

[Example 12] Improvement of pH-Dependent Binding to Membrane-Type IL-6 Receptor by Optimization of Constant Region

Optimization of Constant Region of Fv4-IgG1

Generally, binding to membrane-bound antigens has been reported to vary depending on the constant region of the antibody (J. Immunol. Methods 1997 Jun 23; 205(1): 67-72). The constant regions of the pH-dependent-binding antibodies prepared above were of the IgG1

isotype. Therefore, a study was made for optimization of the constant region in order to improve the pH-dependent binding to the membrane-type IL-6 receptor.

A mutation was introduced into a naturally-occurring constant region, i.e., constant region IgG2 (SEQ ID NO: 28), to construct constant region IgG2ΔGK (SEQ ID NO: 29). Another mutation was introduced into the constant region IgG2ΔGK to construct constant region M58 (SEQ ID NO: 30). Mutations were further introduced into the constant regions IgG2 and M58 to construct constant regions M71 (SEQ ID NO: 31) and M73 (SEQ ID NO: 32).

VH3-IgG2ΔGK (SEQ ID NO: 33) was constructed by substituting the constant region of VH3-IgG1 prepared in Example 10 with IgG2ΔGK, VH3-M58 (SEQ ID NO: 34) was constructed by substituting the constant region with M58, and VH3-M73 (SEQ ID NO: 35) was constructed by substituting the constant region with M73. More specifically, expression vectors in which the constant region portion of VH3 used in Example 10 was substituted by a desired constant region by NheI/NotI digestion and ligation were constructed. The nucleotide sequences of the resulting expression vectors were determined using a method known among persons with ordinary skill in the art.

Expression and purification were carried out for the following: Fv4-IgG2 using VH3-IgG2ΔGK (SEQ ID NO: 33) for the H chain and VL3-CK (SEQ ID NO: 27) for the L chain; Fv4-M58 using VH3-M58 (SEQ ID NO: 34) for the H chain and VL3-CK (SEQ ID NO: 27) for the L chain; and Fv4-M73 using VH3-M73 (SEQ ID NO: 35) for the H chain and VL3-CK (SEQ ID NO: 27) for the L chain. Expression and purification were carried out using the method described in Example 1.

Analysis of Binding of Fv4 Having Optimized Constant Region to Soluble IL-6 Receptor

The association with and dissociation from SR344 were observed on a real-time basis using the same method as Example 10 for thus-prepared Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73 as well as WT. The association rate constants k_a (1/Ms) and dissociation rate constants k_d (1/s) were calculated after analysis in the same manner, and then, dissociation constants KD (M) were calculated on the basis of those values (Table 9).

[Table 9] Comparison of Dissociation Rate Constants of pH-Dependently-Binding Clones from Soluble IL-6 Receptor, SR344

Sample	ka (1/Ms)	kd (1/s)	KD (M)
Fv4-IgG1	7.2E+05	1.0E-03	1.4E-09
Fv4-IgG2	9.6E+05	1.2E-03	1.3E-09
Fv4-M58	8.3E+05	1.1E-03	1.4E-09
Fv4-M73	7.5E+05	1.0E-03	1.4E-09

As a result of calculating the affinity at pH 7.4 for each clone, the dissociation constants (affinity, KD value) of Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73 to SR344 were 1.4 nM, 1.3 nM, 1.4 nM, and 1.4 nM, respectively, and they are almost equivalent. This indicates that the binding ability of pH-dependent-binding clones to the soluble IL-6 receptor, SR344, does not change even after modifying the constant region. On the basis of this finding, the binding ability to the soluble IL-6 receptor was thought to not change for Fv1, Fv2, and Fv3 even if the constant region was similarly modified.

Analysis of Binding of Fv4 Having Optimized Constant Region to Membrane-Type IL-6 Receptor

Antigen-antibody reactions to the membrane-type IL-6 receptor at pH 5.8 and pH 7.4 were observed for thus-prepared Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73 as well as WT, in the same manner as in Example 10 using Biacore T100 (GE Healthcare). The results obtained by injecting the pH-dependent-binding clones under the conditions of pH 7.4 to allow binding to SR344, and by observing the pH-dependent dissociation of each clone in the pH 5.8 mobile phase, are shown in FIG. 19. Further analyses were conducted in the same manner as in Example 10, and the pH-dependent dissociation rates for each clone are shown in Table 10.

[Table 10] Comparison of Dissociation Rate Constants of pH-Dependently-Binding Clones from Membrane-Type IL-6 Receptor, SR344

Sample	pH7.4 kd (1/s)	pH5.8 kd (1/s)	pH Dependency kd(pH5.8)/kd(pH7.4)
Fv4-IgG1	4.7E-04	2.6E-03	5.56
Fv4-IgG2	1.0E-03	1.8E-02	16.99
Fv4-M58	5.4E-04	9.5E-03	17.64
Fv4-M73	5.1E-04	5.1E-03	10.06

As a result of calculating the pH dependency for each clone, the pH dependencies of Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73 to SR344 were 5.6-fold, 17.0-fold, 17.6-fold, and 10.1-fold, respectively; thus, Fv4-IgG2, Fv4-M58, and Fv4-M73 all demonstrated higher pH-dependent dissociation from the membrane-type IL-6 receptor than Fv4-IgG1.

Based on the results of analyzing binding to the soluble IL-6 receptor and binding to the membrane-type IL-6 receptor using the variable region of Fv4, it was found that substitution of the constant region from IgG1 to IgG2, M58, or M73 could improve the pH-dependent binding to the membrane-type IL-6 receptor, without causing a change in the affinity to the soluble IL-6 receptor. This was considered to similarly hold for Fv1, Fv2, and Fv3.

[Example 13] PK/PD Test of pH-Dependently-Binding Antibodies Having Optimized Constant Region Using Human IL-6 Receptor Transgenic Mice

The pharmacokinetics of Fv4-IgG1, Fv4-IgG2, and Fv4-M58 prepared in Example 13 were evaluated using the human IL-6 receptor transgenic mice (hIL-6R tg mice) used in Example 8 to examine the effects of the constant region on the pharmacokinetics. WT, Fv4-IgG1, Fv4-IgG2, or Fv4-M58 was administered to the hIL-6R tg mice by single-dose intravenous administration at 25 mg/kg, and then, measurement of the plasma concentrations of each antibody was carried out in the same manner as in Example 8. The time courses of plasma concentrations of WT, Fv4-IgG1, Fv4-IgG2, and Fv4-M58 are shown in FIG. 20.

Similar to Example 11, the pharmacokinetics of Fv4-IgG1 was improved in comparison with WT, and the pharmacokinetics of Fv4-IgG2 and Fv4-M58 was further improved in comparison with Fv4-IgG1. Measurement as to the unbound IL-6 receptor concentrations, as measured in cynomolgus monkeys in Example 9, was carried out in the hIL-6R tg mice in this test using the same method. As a result, prolongation of the duration of neutralization of the soluble IL-6 receptor was confirmed for Fv4-IgG2 and Fv4-M58 in comparison to that of Fv4-IgG1 (data not shown). As indicated in Example 10, the pH-dependent binding to the membrane-type IL-6 receptor was improved for Fv4-IgG2 and Fv4-M58 as compared with Fv4-IgG1. Therefore, it was shown that improvement in the pH-dependent binding to the membrane-type IL-6 receptor and improvement in the pharmacokinetics and duration of neutralization of the soluble IL-6 receptor are possible by substituting the constant region from IgG1 to IgG2 or M58. On the basis of this finding, it was thought that the pharmacokinetics and duration of neutralization of the soluble IL-6 receptor, not only in the case of Fv4, but also in the cases of Fv1, Fv2, and Fv3, can be improved as compared to IgG1 by substituting the constant region from IgG1 to IgG2 or M58.

[Example 14] Construction of pH-Dependently-Binding Antibodies Having Optimized Variable and Constant Regions

VH2-M71 (SEQ ID NO: 36) and VH2-M73 (SEQ ID NO: 37), having M71 and M73 for the constant region of VH2-IgG1, and VH4-M71 (SEQ ID NO: 38) and VH4-M73 (SEQ ID NO: 39), having M71 and M73 for the constant region of VH4-IgG1, were constructed using the same method as described above.

Fv1-M71 using VH2-M71 for the H chain and VL2-CK for the L chain, Fv1-M73 using VH2-M73 for the H chain and VL2-CK for the H chain, Fv3-M71 using VH4-M71 for the H chain and VL1-CK for the L chain, and Fv3-M73 using VH4-M73 for the H chain and VL1-CK

for the L chain, were expressed and purified. Expression and purification were carried out using the method described in Example 1.

Analyses of Binding of pH-Dependent-Binding Antibodies Having Optimized Variable and Constant Regions to Soluble IL-6 Receptor

The association to and dissociation from SR344 were observed on a real-time basis using the same method as Example 10 for the eleven types of antibodies, humanized PM1 antibody (wild type: WT) and H3pI/L73-IgG1, Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M71, Fv3-M73, Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73, constructed as described above. The association rate constants k_a (1/Ms) and dissociation rate constants k_d (1/s) were calculated by analysis in the same manner, and the dissociation constants KD (M) were calculated on the basis of those values (Table 11).

[Table 11] Comparison of Dissociation Rate Constants of pH-Dependently-Binding Clones from Soluble IL-6 Receptor, SR344

Sample	k_a (1/Ms)	k_d (1/s)	KD (M)
WT	4.0E+05	1.1E-03	2.7E-09
H3pI/L73	4.1E+05	5.9E-04	1.4E-09
Fv1-M71	5.5E+05	5.4E-04	9.7E-10
Fv1-M73	6.1E+05	5.5E-04	9.1E-10
Fv2-IgG1	3.9E+05	7.7E-04	2.0E-09
Fv3-M71	7.8E+05	8.2E-04	1.1E-09
Fv3-M73	8.5E+05	8.7E-04	1.0E-09
Fv4-IgG1	7.2E+05	1.0E-03	1.4E-09
Fv4-IgG2	9.6E+05	1.2E-03	1.3E-09
Fv4-M58	8.3E+05	1.1E-03	1.4E-09
Fv4-M73	7.5E+05	1.0E-03	1.4E-09

All of the resulting ten types of pH-dependent-binding clones were found to have dissociation constants (affinity, KD values) to the soluble IL-6 receptor equal to or stronger than that of WT.

Analyses of Binding of pH-Dependently-Binding Antibodies Having Optimized Variable and Constant Regions to Membrane-Type IL-6 Receptor

Antigen-antibody reactions to the membrane-type IL-6 receptor at pH 5.8 and pH 7.4 were observed in the same manner as in Example 10 using Biacore T100 (GE Healthcare) for the eleven types of antibodies, humanized PM1 antibody (wild type: WT) and H3pI/L73-IgG1, Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M71, Fv3-M73, Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73, prepared as described above. The pH-dependently-binding clones were injected

under the condition of pH 7.4 to allow them to bind to SR344, and then, the pH-dependent dissociation of each clone at the pH of the mobile phase, pH 5.8, was observed. The results are shown in FIG. 21 (results for Fv1-M71, Fv1-M73, Fv3-M71, and Fv3-M73 are shown in FIG. 21, while results for other clones are shown in FIGs. 17 and 19). Analyses were carried out in the same manner as in Example 10 and the pH dependencies of the dissociation rate constants of all of the eleven types of clones are shown in Table 12.

[Table 12] pH Dependencies of Dissociation Rate Constants of pH-Dependently-Binding Clones from Membrane-Type IL-6 Receptor, SR344

Sample	pH7.4 kd (1/s)	pH5.8 kd (1/s)	pH Dependency kd(pH5.8)/kd(pH7.4)
WT	2.5E-04	2.5E-04	1.00
H3pI/L73	2.6E-04	6.7E-04	2.59
Fv1-M71	6.1E-04	6.9E-03	11.29
Fv1-M73	3.7E-04	3.2E-03	8.80
Fv2-IgG1	3.4E-04	2.4E-03	7.18
Fv3-M71	9.1E-04	9.7E-03	10.74
Fv3-M73	4.9E-04	5.3E-03	10.88
Fv4-IgG1	4.7E-04	2.6E-03	5.56
Fv4-IgG2	1.0E-03	1.8E-02	16.99
Fv4-M58	5.4E-04	9.5E-03	17.64
Fv4-M73	5.1E-04	5.1E-03	10.06

The ten types of obtained pH-dependently-binding clones demonstrated pH-dependent binding ability to the membrane-type IL-6 receptor. Moreover, all of Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M71, Fv3-M73, Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73 were found to demonstrate improved pH-dependent binding to the membrane-type IL-6 receptor, in comparison with H3pI/L73-IgG1, for which the time until the antibody disappears from the plasma as well as the time where the soluble IL-6 receptor and membrane-type IL-6 receptor are bound by the antibody in the body were found to be prolonged considerably in comparison with WT, as shown in cynomolgus monkeys in Example 9.

[Example 15] PK/PD Test of pH-Dependently-Binding Antibodies Having Optimized Variable and Constant Regions Using Cynomolgus Monkeys

Construction of Known High-Affinity Anti-IL-6 Receptor Antibody

A mammalian cell expression vector was constructed to express the high-affinity anti-IL-6 receptor antibody VQ8F11-21 hIgG1 described in US 2007/0280945 A1 (US 2007/0280945 A1, amino acid sequences 19 and 27), as a known high-affinity anti-IL-6 receptor antibody. An antibody variable region was constructed by PCR combining synthetic oligo-DNAs (assembly PCR). The constant region was amplified by PCR from the expression vector used in Example 1. The antibody variable region and antibody constant region were

ligated by assembly PCR and inserted into a vector for expression in mammals. The resulting H chain and L chain DNA fragments were inserted into mammalian cell expression vectors to construct the H chain expression vector and L chain expression vector of interest. The nucleotide sequences of the resulting expression vectors were determined by a method known among persons with ordinary skill in the art. Expression and purification were carried out using the constructed expression vectors. Expression and purification were carried out using the method described in Example 1 to obtain the high-affinity anti-IL-6 receptor antibody (high affinity Ab).

PK/PD Test in Cynomolgus Monkeys

The pharmacokinetics and pharmacological efficacy were evaluated in cynomolgus monkeys for the pH-dependently-binding antibodies H3pI/L73-IgG1 and Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M73, and Fv4-M73 and the known high-affinity anti-IL-6 receptor antibody (high affinity Ab). H3pI/L73-IgG1, Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M73, or Fv4-M73 was administered to cynomolgus monkeys by single-dose intravenous administration at 0.5 mg/kg, while the high affinity Ab was administered by single-dose intravenous administration at 1.0 mg/kg. Blood samples were collected before administration and over time. The plasma concentration of each antibody was measured in the same manner as in Example 9. The time courses of plasma concentrations of H3pI/L73-IgG1, Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M73, and Fv4-M73, as well as the high affinity Ab are shown in FIG. 21. In order to evaluate the pharmacological efficacy in terms of the degree to which the cynomolgus monkey membrane-type IL-6 receptor is neutralized, cynomolgus monkey IL-6 was administered subcutaneously into the lower backs of the animals daily at 5 μg/kg from day 3 to day 10 (from day 6 to day 10 with respect to the high affinity Ab) after the administration of antibody, in the same manner as in Example 9. The CRP concentration of each animal was measured 24 hours after each administration. The time courses of CRP concentrations with the administration of each antibody are shown in FIG. 22. In order to evaluate the pharmacological efficacy in terms of the degree of neutralization of cynomolgus monkey soluble IL-6 receptor, the concentration of the unbound cynomolgus monkey soluble IL-6 receptor in cynomolgus monkey plasma was measured in the same manner as Example 9. The time courses of the unbound cynomolgus monkey soluble IL-6 receptor concentrations with the administration of each antibody are shown in FIG. 23.

As a result, the antibody concentrations in plasma were maintained high for each of Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M73 and Fv4-M73 in comparison with H3pI/L73-IgG1, while the concentrations of CRP and the unbound cynomolgus monkey soluble IL-6 receptor were maintained at low levels. Namely, this result showed that the time where the

membrane-type and soluble IL-6 receptors are bound by the antibody (or in other words, the duration of neutralization) was prolonged by the antibodies in comparison with H3pI/L73-IgG1.

In addition, these pH-dependently-binding anti-IL-6 receptor antibodies were confirmed for their neutralization effects and sustained efficacy equal to or greater than that of the known high-affinity anti-IL-6 receptor antibody (high affinity Ab) administered at 1.0 mg/kg, at only half the dosage thereof, i.e., at 0.5 mg/kg. Therefore, the pH-dependently-binding antibodies were elucidated to have the neutralization effects and sustained efficacy superior to those of the known high-affinity anti-IL-6 receptor antibody.

Those antibodies shown in Table 12 for which PK/PD tests were not carried out using cynomolgus monkeys as in this test, have also been confirmed to demonstrate improved pH-dependent binding to the membrane-type IL-6 receptor in comparison with H3pI/L73-IgG1. Therefore, the time during which the membrane-type and soluble IL-6 receptor are bound by the antibodies (or in other words, the duration of neutralization and sustained neutralization effects) are also thought to be prolonged for the antibodies in comparison with H3pI/L73-IgG1.

In Example 9, for H3pI/L73-IgG1, the time until the antibody disappears from the plasma as well as the time where the soluble IL-6 receptor and membrane-type IL-6 receptor are bound by the antibody in the body (sustained neutralization effects) were found to be prolonged considerably as compared with WT. Fv1-M71, Fv1-M73, Fv2-IgG1, Fv3-M71, Fv3-M73, Fv4-IgG1, Fv4-IgG2, Fv4-M58, and Fv4-M73, having superior sustained neutralization effects to H3pI/L73-IgG1, are therefore thought to have remarkably improved sustained neutralization effects as compared with WT.

In contrast to anti-IL-6 receptor antibodies, the pH-dependent-binding anti-IL-6 receptor antibodies that are made to strongly bind to the antigen at the pH in the plasma of pH 7.4 but only weakly bind to the antigen at the pH in endosomes of pH 5.8, make it possible to reduce the patient dosage and administration frequency of the anti-IL-6 receptor antibody, and as a result, they can considerably reduce the total administration amount. Therefore, the pH-dependently-binding anti-IL-6 receptor antibodies are thought to be extremely superior as a pharmaceutical for use as an IL-6 antagonist.

[Example 16] Construction of pH-Dependently Binding Anti-IL-6 Antibody

Expression and Purification of Anti-IL-6 Antibody

In Examples 1 to 15, a plurality of humanized anti-IL-6 receptor antibodies that pH-dependently bind to the IL-6 receptor were successfully created by imparting the dependency through introducing histidine substitutions and the like into the variable region, in particular, the CDR sequences of the humanized anti-IL-6 receptor antibodies. It was found that all of these

antibodies repeatedly bind to the IL-6 receptor and demonstrate a considerable improvement in PK/PD.

Therefore, it was confirmed that the pH-dependent ability of an antibody to bind to an antigen could be confered to another antibody that binds to an antigen other than the IL-6 receptor using a similar method. Human IL-6 was selected as the antigen, and an anti-IL-6 antibody including the H chain (WT) (amino acid sequence: SEQ ID NO: 62) and L chain (WT) (amino acid sequence: SEQ ID NO: 63), which binds to IL-6 as described in WO 2004/039826, ("Anti-IL6 wild type") was constructed. Using a method known to those skilled in the art, gene fragments encoding the antibody amino acid sequences of interest were inserted into mammalian cell expression vectors to construct the H chain expression vector and L chain expression vector of interest. The nucleotide sequences of the resulting expression vectors were determined using a method known to a skilled person. Anti-IL6 wild type was expressed and purified by the method described in Example 1.

Construction of pH-Dependent Anti-IL-6 Antibody

To confer the pH-dependent ability of the antibody to bind to IL-6, histidine substitutions were introduced into the amino acids in CDR of the anti-IL-6 antibody (Anti-IL6 wild type) including the H chain (WT) (amino acid sequence: SEQ ID NO: 62) and L chain (WT) (amino acid sequence: SEQ ID NO: 63). By substituting histidine in the CDR amino acids and subsequently screening, several clones that demonstrate the pH-dependent binding were obtained. The binding at pH 5.5 was significantly reduced as compared with the binding at pH 7.4. The positions of histidine substitution in the pH-dependent clones are shown in Table 13. The examples include "Anti-IL6 clone 1" including the H chain (c1) (amino acid sequence: SEQ ID NO: 64) and L chain (c1) (amino acid sequence: SEQ ID NO: 65), and "Anti-IL-6 clone 2" including the H chain (c1) (amino acid sequence: SEQ ID NO: 64) and L chain (c2) (amino acid sequence: SEQ ID NO: 66). Anti-IL6 clone 1 and Anti-IL-6 clone 2 were expressed and purified by the method described in Example 1.

[Table 13] Positions of Histidine Substitution in pH-dependent clones

H32, H59, H61, H99	
L53, L54, L90, L94	

Analysis of Binding of pH-Dependent Clones to Human IL-6

Kinetic analysis of antigen-antibody reactions at pH 5.5 and pH 7.4 was carried out using Biacore T100 (GE Healthcare) for the three types of antibodies prepared as mentioned above: Anti-IL6 wild type, Anti-IL6 clone 1, and Anti-IL-6 clone 2 (buffer: DPBS(-) (pH 7.4 or pH 5.5),

150 mM NaCl). The antibodies were bound to a sensor chip on which recomb-protein A/G (Pierce) was immobilized by amine coupling, and then human IL-6 (Toray) adjusted to an appropriate concentration was injected on the chip as an analyte. All the measurements were carried out at 37°C. Association rate constants k_a (1/Ms) and dissociation rate constants k_d (1/s) were calculated using Biacore T100 Evaluation Software (GE Healthcare), and dissociation constants KD (M) were calculated based on those values (Table 14). Furthermore, the ratio of affinity at pH 5.5 and pH 7.4 was calculated for each clone to evaluate the pH-dependent binding.

[Table 14] Comparison of Binding of pH-Dependent Clones to IL-6

Sample	pH	ka(1/Ms)	kd(1/s)	KD(M)	KD(pH5.5)/KD(pH7.4)
Wild type	pH7.4	2.05E+07	3.91E-04	1.91E-11	0.8
	pH5.5	1.52E+07	2.45E-04	1.61E-11	
clone1	pH7.4	1.07E+07	4.71E-03	4.38E-10	10.3
	pH5.5	2.05E+06	9.26E-03	4.52E-09	
clone2	pH7.4	8.96E+06	2.63E-03	2.94E-10	13.5
	pH5.5	2.76E+06	1.10E-02	3.98E-09	

The ratio of affinity at pH 5.5 and pH 7.4 ((KD)(pH 5.5)/(KD)(pH 7.4)) calculated, which indicates the pH-dependent binding to human IL-6, was 0.8, 10.3, and 13.5 for Anti-IL6 wild type, Anti-IL6 clone 1, and Anti-IL6 clone 2, respectively. That is, the pH-dependent binding ability of each clone is more than 10 times greater than that of WT. The sensorgrams of Anti-IL-6 clone 2 at pH 7.4 and pH 5.5 are shown in FIG. 26.

Thus, it was shown that, as in the case of the anti-IL-6 receptor antibodies, pH-dependently binding anti-IL-6 antibodies that bind to the antigen strongly under plasma neutral conditions, but weakly under intraendosomal acidic conditions can be constructed by introducing histidine substitutions and the like mainly into the CDR amino acid sequences. As indicated in Examples 1 to 15, an anti-IL-6 receptor antibody that has the pH-dependent binding ability repeatedly binds to the IL-6 receptor and PK/PD is remarkably improved. That is, it was suggested that Anti-IL-6 clone 1 and Anti-IL-6 clone 2, which have the pH-dependent binding ability, repeatedly bind to more antigens with significantly improved PK/PD, as compared with Anti-IL6 wild type.

[Example 17] Construction of pH-Dependently Binding Anti-IL-31 Receptor Antibody

Expression and Purification of Anti-IL-31 Receptor Antibody

In Examples 1 to 15, a plurality of humanized anti-IL-6 receptor antibodies that pH-dependently bind to the IL-6 receptor were successfully created by conferring the pH

dependency through introducing histidine substitutions and the like into the variable region, in particular, the CDR sequences of the humanized anti-IL-6 receptor antibodies. It was found that all of these antibodies repeatedly bind to the IL-6 receptor and demonstrate a considerable improvement of PK/PD.

Therefore, it was confirmed that the pH dependent ability of an antibody to bind to an antigen could be confered to another antibody that binds to an antigen other than the IL-6 receptor using a similar method. Mouse IL-31 receptor was selected as the antigen, and an anti-IL-31 receptor antibody including the H chain (WT) (amino acid sequence: SEQ ID NO: 67) and L chain (WT) (amino acid sequence: SEQ ID NO: 68), which binds to the mouse IL-31 receptor as described in WO 2007/142325, ("Anti-IL31R wild type") was constructed. Using a method known to those skilled in the art, gene fragments encoding the amino acid sequences of interest were inserted into mammalian cell expression vectors to construct the H chain expression vector and L chain expression vector of interest. The nucleotide sequences of the resulting expression vectors were determined using a method known to a skilled person. Anti-IL31R wild type was expressed and purified by the method described in Example 1.

Construction of pH-Dependent Anti-IL-31 Receptor Antibody

To confer the pH-dependent ability of the antibody to bind to the IL-31 receptor, histidine substitutions were introduced into the amino acids of CDR of the anti-IL-31 receptor antibody (Anti-IL31R wild type) including the H chain (WT) (amino acid sequence: SEQ ID NO: 67) and L chain (WT) (amino acid sequence: SEQ ID NO: 68). By histidine substitutions in the CDR amino acids and subsequent screening, several clones that demonstrate the pH-dependent binding were obtained. The binding at pH 5.5 was significantly reduced as compared with the binding at pH 7.4. The position of histidine substitution in the pH-dependent clones is shown in Table 15. An example is "Anti-IL31R clone 1" including the H chain (c1) (amino acid sequence: SEQ ID NO: 69) and L chain (WT). Anti-IL31R clone 1 was expressed and purified using the method described in Example 1.

[Table 15] Position of Histidine Substitution in pH-dependent clones

H33

Analysis of Binding of pH-Dependent Clones to Soluble IL-31 Receptor

Kinetic analysis of antigen-antibody reactions at pH 5.5 and pH 7.4 was carried out using Biacore T100 (GE Healthcare) for the two types of antibodies prepared as mentioned above: Anti-IL31R wild type and Anti-IL31R clone 1 (buffer: DPBS(-) (pH 7.4 or pH 5.5), 150 mM NaCl, 0.01% Tween 20, 0.02% NaN_3). The antibodies were bound to a sensor chip on which

recomb-protein A/G (Pierce) was immobilized by amine coupling, and then the soluble mouse IL-31 receptor (prepared according to the method described in WO 2007/142325) adjusted to an appropriate concentration was injected therein as an analyte. All the measurements were carried out at 25°C. Association rate constants k_a (1/Ms) and dissociation rate constants k_d (1/s) were calculated using Biacore T100 Evaluation Software (GE Healthcare), and dissociation constants KD (M) were calculated based on those values (Table 16). Furthermore, the ratio of affinity at pH 5.5 and pH 7.4 was calculated for each clone to evaluate the pH-dependent binding.

[Table 16] Comparison of Binding of pH-Dependent Clones to Mouse IL-31 Receptor

Sample	pH	ka(1/Ms)	kd(1/s)	KD(M)	KD(pH5.5)/KD(pH7.4)
Wild type	pH7.4	1.40E+05	3.40E-03	2.30E-08	3.2
	pH5.5	5.10E+05	3.80E-03	7.40E-08	
clone1	pH7.4	1.70E+05	3.30E-03	2.20E-08	1000.0
	pH5.5	1.10E+03	2.40E-02	2.20E-05	

The ratio of affinity at pH 5.5 and pH 7.4 ((KD)(pH 5.5)/(KD)(pH 7.4)) calculated, which indicates the pH-dependent binding to the mouse IL-31 receptor, was 3.2 and 1000 for Anti-IL31R wild type and Anti-IL31R clone 1, respectively. That is, the pH-dependent binding ability of Anti-IL31R clone 1 is about 300 times greater than that of WT. The sensorgrams for Anti-IL31R clone 1 at pH 7.4 and pH 5.5 are shown in FIG. 27.

Thus, it was shown that, as in the cases of the anti-IL-6 receptor antibodies and anti-IL-6 antibodies, pH-dependent binding anti-IL-31 receptor antibodies that bind to the antigen strongly under plasma neutral conditions, but weakly under intraendosomal acidic conditions can be constructed by introducing histidine substitutions and the like mainly into the CDR amino acid sequences. As indicated in Examples 1 to 15, an anti-IL-6 receptor antibody that has the pH-dependent binding ability repeatedly binds to the IL-6 receptor and PK/PD is remarkably improved. That is, it was suggested that Anti-IL31R clone 1, which has the pH-dependent binding ability, repeatedly binds to more antigens with significantly improved PK/PD, as compared with Anti-IL31R wild type.

[Example 18] Repetitive Binding to Antigen by pH-Dependently Binding Antibody

Expression and Purification of Antibody Administered to Mice

The four types of humanized IL-6 receptor antibodies described below were prepared. As the antibodies that do not pH-dependently bind to the IL-6 receptor, WT-IgG1 including H

(WT) (amino acid sequence: SEQ ID NO: 9) and L (WT) (amino acid sequence: SEQ ID NO: 10), and H54/L28-IgG1 including H54 (amino acid sequence: SEQ ID NO: 70) and L28 (amino acid sequence: SEQ ID NO: 12) were expressed and purified using the method indicated in Example 1. As the antibodies that pH-dependently bind to the IL-6 receptor, H170/L82-IgG1 of Example 3 including H170 (amino acid sequence: SEQ ID NO: 4) and L82 (amino acid sequence: SEQ ID NO: 7), and Fv4-IgG1 of Example 10 including VH3-IgG1 (SEQ ID NO: 23) and VL3-CK (SEQ ID NO: 27), were expressed and purified using the method indicated in Example 1.

Analysis of Binding of Each Type of Antibody to Soluble IL-6 Receptor

Kinetic analysis of antigen-antibody reactions at pH 7.4 and pH 5.8 was carried out using Biacore T100 (GE Healthcare) for the four types of antibodies prepared: WT-IgG1, H54/L28-IgG1, H170/L82-IgG1, and Fv4-IgG1 (buffer: 10 mM MES (pH 7.4 or pH 5.8), 150 mM NaCl, 0.05% Surfactant-P20). The antibodies were bound to a sensor chip on which recomb-protein A/G (Pierce) was immobilized by amine coupling, and SR344 adjusted to an appropriate concentration was injected therein as an analyte. The association with and dissociation from SR344 of each type of antibody were observed on a real-time basis. All the measurements were carried out at 37°C. Association rate constants k_a (1/Ms) and dissociation rate constants k_d (1/s) were calculated using Biacore T100 Evaluation Software (GE Healthcare), and dissociation constants KD (M) were calculated based on those values (Table 17).

[Table 17] Comparison of Association Rates (k_a), Dissociation Rates (k_d), and Dissociation Constants of Each Type of Antibody Against Soluble IL-6 Receptor (SR344)

Sample	pH7.4			pH5.8			pH Dependency	
	ka(1/Ms)	kd(1/s)	KD(M)	ka(1/Ms)	kd(1/s)	KD(M)	kd(pH5.8)/kd(pH7.4)	KD(pH5.8)/KD(pH7.4)
WT-IgG1	4.9E+05	9.4E-04	1.9E-09	8.9E+05	2.7E-03	3.1E-09	2.9	1.6
H54/L28-IgG1	8.3E+05	1.4E-03	1.7E-09	2.4E+06	2.7E-03	1.1E-09	2.0	0.7
H170/L82-IgG1	6.7E+05	1.1E-03	1.6E-09	1.2E+05	1.3E-02	1.0E-07	11.4	61.9
Fv4-IgG1	9.8E+05	9.5E-04	9.7E-10	1.4E+06	3.7E-02	2.6E-08	38.8	27.3

The ratio of affinity (KD) at pH 5.8 and pH 7.4 for each antibody was calculated. The KD ratio, which indicates the pH-dependent binding to SR344, was 1.6, 0.7, 61.9, and 27.3 for WT-IgG1, H54/L28-IgG1, H170/L82-IgG1, and Fv4-IgG1, respectively. In addition, the ratio of dissociation rate (k_d) at pH 5.8 and pH 7.4 for each antibody was calculated. The k_d ratio, which indicates the pH-dependent dissociation rate for SR344, was 2.9, 2.0, 11.4, and 38.8 for WT-IgG1, H54/L28-IgG1, H170/L82-IgG1, and Fv4-IgG1, respectively. Thus, it was confirmed that H170/L82-IgG1 and Fv4-IgG1 demonstrate the pH-dependent binding, while the conventional antibodies WT-IgG1 and H54/L28-IgG1 hardly exhibit the ability. In addition,

since the affinity (KD) of these antibodies at pH 7.4 was nearly equal, their ability to bind to SR344 in the plasma was thought to be equivalent.

In Vivo Pharmacokinetics Test Using Mice

The pharmacokinetics of SR344 and the anti-human IL-6 receptor antibody were evaluated following administration of SR344 (human IL-6 receptor, prepared in Example 1) only or simultaneous administration of SR344 and the anti-human IL-6 receptor antibody to mice that do not express the human IL-6 receptor (C57BL/6J; the anti-human IL-6 receptor antibodies do not bind to the mouse IL-6 receptor). An SR344 solution (5 μg/mL) or a mixed solution containing SR344 and the anti-human IL-6 receptor antibody (5 μg/mL and 0.1 mg/mL, respectively) was administered into a caudal vein by single-dose administration at 10 mL/kg. Since the anti-human IL-6 receptor antibody was present in an adequate excess amount relative to SR344, it was thought that nearly all of the SR344 molecules were bound by the antibody. Blood samples were collected at 15 minutes, 2 hours, 8 hours, 1 day, 2 days, 3 days, 4 days, 7 days, 14 days, 21 days, and 28 days after administration. The collected blood samples were immediately centrifuged for 15 minutes at 15,000 rpm and 4°C to obtain the plasma. The plasma separated was stored in a freezer set to -20°C or lower until the time of measurement. Above-described WT-IgG1, H54/L28-IgG1, H170/L82-IgG1, and Fv4-IgG1 were used as the anti-human IL-6 receptor antibodies.

Measurement of Anti-Human IL-6 Receptor Antibody Plasma Concentration by ELISA

The concentration of 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 SR344 was added to 100 μL of the calibration curve samples and plasma samples, and then the samples were allowed to stand for 1 hour at room temperature. Subsequently, the samples were dispensed into the anti-human IgG-immobilized plates, and allowed to stand for 1 hour at room temperature. Then, Biotinylated Anti-Human IL-6R Antibody (R&D) was added to react for 1 hour at room temperature. Subsequently, Streptavidin-PolyHRP80 (Stereospecific Detection Technologies) was added to react for 1 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 1 N 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. 28.

Measurement of SR344 Plasma Concentration by Electrochemiluminescence

The concentration of SR344 in mouse plasma was measured by electrochemiluminescence. SR344 calibration curve samples adjusted to concentrations of 2000, 1000, 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 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 SR344 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 1 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 SR344 concentration was calculated based on the response of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices). The time course of SR344 plasma concentration after intravenous administration as measured by this method is shown in FIG. 29.

Effects of pH-Dependent Binding

With respect to the time course of antibody concentration of WT-IgG1 and H54/L28-IgG1, which do not demonstrate the pH-dependent binding, and H170/L82-IgG1 and Fv4-IgG1, which demonstrate the pH-dependent binding, the time course of concentration was roughly identical for WT-IgG1, H54/L28-IgG1 and Fv4-IgG1, while H170/L82-IgG1 was eliminated slightly more rapidly. The data of the time course of plasma concentration was analyzed by the pharmacokinetics analysis software WinNonlin (Pharsight). The half-lives in the plasma of WT-IgG1, H54/L28-IgG1, Fv4-IgG1, and H170/L28-IgG1 were 21.0, 28.8, 26.2, and 7.5 days, respectively.

As described in Example 2, when the antigen is a soluble antigen, an administered antibody binds to the antigen in the plasma, and is retained in the plasma in the form of an antigen-antibody complex. Generally, in contrast to extremely long plasma retention time of an antibody (the elimination rate is extremely low) due to the function of FcRn, the plasma retention time of an antigen is short (the elimination rate is high). Thus, an antigen that is

bound to an antibody has a prolonged plasma retention time similar to that of an antibody (the elimination rate is extremely low). Similarly, when the antigen of humanized IL-6 receptor antibody, SR344 (soluble human IL-6 receptor), was administered alone, SR344 was extremely rapidly eliminated (plasma half-life: 0.2 days). However, in the case of concurrent administration of SR344 with a conventional antibody, WT-IgG1 or H54/L28-IgG1, which does not demonstrate the pH-dependent binding, the elimination rate of SR344 was considerably reduced, and the plasma retention time of SR344 was prolonged (plasma half-life: 5.3 days for WT-IgG1, 6.3 days for H54/L28-IgG1). This is because nearly all of the SR344 molecules were bound by the antibodies administered together, and thus SR344 bound by the antibodies had a prolonged plasma retention time similar to that of the antibody, due to the function of FcRn as described above.

In the case of concurrent administration of SR344 with the H170/L82-IgG1 or Fv4-IgG1 antibody, which demonstrates the pH-dependent binding, the elimination of SR344 was significantly rapid (plasma half-life: 1.3 days for H170/L82-IgG1, 0.6 days for Fv4-IgG1), as compared to the case of concurrent administration with WT-IgG1 or H54/L28-IgG1. This tendency was particularly prominent for Fv4-IgG1. Since the affinity of Fv4-IgG1 at pH 7.4 is equivalent to or stonger than that of WT-IgG1 and H54/L28-IgG1, it is thought that nearly all of the SR344 molecules were bound to Fv4-IgG1. Even though Fv4-IgG1 demonstrates equivalent or slightly longer plasma retention and slower elimination compared to WT-IgG1 and H54/L28-IgG1, the elimination of SR344 bound to Fv4-IgG1 was extremely rapid. This can be explained by the concept of the present technology shown in FIG. 4. In the case of conventional antibodies that do not demonstrate the pH-dependent binding, an antibody-soluble antigen complex is taken up into endosomes by pinocytosis in the plasma, and binds to FcRn expressed in endosomes under intraendosomal acidic conditions. Since the antibody-soluble antigen complex bound to FcRn transfers to the cell surface as it is, and again returns to the plasma, the antigen bound to the antibody has a prolonged plasma retention time similar to that of the antibody (the elimination is extremely slow). On the other hand, in the case of antibodies that demonstrate the pH-dependent binding, the antigen dissociates from the antibody under intraendosomal acidic conditions, and thus only the antibody binds to FcRn and returns again to the plasma. Since the antigen dissociated from the antibody is degraded in lysosomes without returning to the plasma, the elimination of antigen is extremely rapid as compared to the case of antibodies that do not demonstrate the pH-dependent binding. Namely, in the case of concurrent administration of SR344 with the WT-IgG1 or H54/L28-IgG1 antibody, which does not demonstrate the pH-dependent binding, the elimination of SR344 is slow to the similar degree as the antibody, since SR344 binds to WT-IgG1 or H54/L28-IgG1 both in plasma and endosomes. In contrast, in the case of concurrent administration of SR344 with the

H170/L82-IgG1 or Fv4-IgG1 antibody, which demonstrates the pH-dependent binding, the elimination of SR344 is extremely rapid, since SR344 dissociates from the antibody in the intraendosomal low-pH environment. That is, since the H170/L28-IgG1 and Fv4-IgG1 antibodies, which demonstrate the pH-dependent binding, dissociate from SR344 in the intraendosomal low-pH environment, the majority of H170/L82-IgG1 or Fv4-IgG1 that has returned again to the plasma with FcRn is thought to be not bound to SR344. Thus, as shown in FIG. 4, it was revealed that, by dissociating from an antigen in the intraendosomal low-pH environment and returning to the plasma with FcRn without binding to the antigen, an antibody that demonstrates the pH-dependent binding can again bind to an antigen in the plasma. It was also shown that, by repeating this process, the antibody that demonstrates the pH-dependent binding can repeatedly bind to multiple antigens. This is consistent with the Biacore data shown in Example 7, demonstrating that pH-dependent antibodies can repeatedly bind to antigens. Thus, by enhancing the pH-dependent binding of an antibody to an antigen, the number of times of repetitive antigen binding can be increased.

When the antigen is a soluble antigen, and the antigen binds to an antibody under plasma neutral conditions, but dissociates from the antibody in endosomes and the antibody returns to the plasma with FcRn, the antibody can again bind to an antigen under plasma neutral conditions. Thus, an antibody that has the ability to dissociate from an antigen under intraendosomal acidic conditions can bind to antigens multiple times. As compared to when an antigen bound to an antibody does not dissociate from the antibody in endosomes (*i.e.*, the antigen bound to the antibody returns to the plasma), if an antigen bound to an antibody dissociates from the antibody in endosomes, the plasma elimination rate of the antigen is increased, since the antigen is transported to lysosomes and degraded. Thus, the plasma elimination rate of an antigen can be used as an index to determine whether an antibody can bind to the antigen multiple times. Determination of the plasma elimination rate of an antigen can be performed, for example, by administering an antigen and an antibody *in vivo*, and then measuring the plasma antigen concentration after the administration, as shown in the Examples.

An antibody that demonstrates the pH-dependent binding can repeatedly bind to multiple antigens in contrast to the case of a conventional antibody that does not demonstrate the pH-dependent binding. Thus, the amount of antibody administered can be considerably reduced, and the administration intervals can be greatly prolonged.

The repetitive binding to multiple antigens of this mechanism is based on pH-dependent antigen-antibody reaction. Thus, regardless of the type of antigen, if an antibody demonstrating the pH-dependent binding that binds to an antigen at pH 7.4 of plasma but dissociates from the antigen at intraendosomal acidic pH can be constructed, then such an antibody can repeatedly bind to multiple antigens. Accordingly, the present technology is useful in that it can be

applied not only to antibodies to the IL-6 receptor, IL-6, and the IL-31 receptor, but generally to any antibody to any antigen, regardless of the type of antigen.

CLAIMS

1. An antigen-binding molecule having a KD(pH5.8)/KD(pH7.4) value, defined as the ratio of KD for the antigen at pH 5.8 and KD for the antigen at pH 7.4, of 2 or higher.

2. The antigen-binding molecule of claim 1, wherein the KD(pH5.8)/KD(pH7.4) value is 10 or higher.

3. The antigen-binding molecule of claim 1, wherein the KD(pH5.8)/KD(pH7.4) value is 40 or higher.

4. The antigen-binding molecule of any one of claims 1 to 3, wherein at least one amino acid of the antigen-binding molecule has been substituted with histidine, or at least one histidine has been inserted into the antigen-binding molecule.

5. The antigen-binding molecule of any one of claims 1 to 4, wherein the antigen-binding molecule has an antagonistic activity.

6. The antigen-binding molecule of any one of claims 1 to 5, wherein the antigen-binding molecule binds to a membrane antigen or a soluble antigen.

7. The antigen-binding molecule of any one of claims 1 to 6, wherein the antigen-binding molecule is an antibody.

8. A pharmaceutical composition comprising the antigen-binding molecule of any one of claims 1 to 7.

9. A method for improving the pharmacokinetics of an antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4.

10. A method for increasing the number of times of antigen-binding for an antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4.

11. A method for increasing the number of antigens that can be bound by an antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4.

12. A method for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4.

13. A method for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4.

14. A method for increasing the ability of an antigen-binding molecule to eliminate an antigen in plasma by impairing the antigen-binding activity of the antigen-binding molecule at pH 5.8 as compared to that at pH 7.4.

15. The method of any one of claims 9 to 14, wherein the KD(pH5.8)/KD(pH7.4) value, defined as the ratio of KD for the antigen at pH 5.8 and KD for the antigen at pH 7.4, is 2 or higher.

16. The method of any one of claims 9 to 14, wherein the KD(pH5.8)/KD(pH7.4) value is 10 or higher.

17. The method of any one of claims 9 to 14, wherein the KD(pH5.8)/KD(pH7.4) value is 40 or higher.

18. A method for improving the pharmacokinetics of an antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.

19. A method for increasing the number of times of antigen-binding for an antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.

20. A method for increasing the number of antigens that can be bound by an antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.

21. A method for dissociating within a cell an antigen from an extracellularly-bound antigen-binding molecule by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.

22. A method for releasing an antigen-binding molecule, which has been bound to an antigen and internalized into a cell, in an antigen-free form to the outside of the cell, by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.

23. A method for increasing the ability of an antigen-binding molecule to eliminate an antigen in plasma by substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.

24. The method of any one of claims 18 to 23, wherein the histidine substitution or insertion increases the KD(pH5.8)/KD(pH7.4) value, defined as the ratio of the antigen-binding activity at pH 5.8 and the antigen-binding activity at pH 7.4, as compared to the KD(pH5.8)/KD(pH7.4) value before the histidine substitution or insertion.

25. The method of any one of claims 9 to 24, wherein the antigen-binding molecule has an antagonistic activity.

26. The method of any one of claims 9 to 25, wherein the antigen-binding molecule binds to a membrane antigen or a soluble antigen.

27. The method of any one of claims 9 to 26, wherein the antigen-binding molecule is an antibody.

28. A method of screening for an antigen-binding molecule, which comprises the steps of:
(a) determining the antigen-binding activity of an antigen-binding molecule at pH 6.7 to pH 10.0;
(b) determining the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5; and
(c) selecting an antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than the antigen-binding activity at pH 4.0 to pH 6.5.

29. The screening method of claim 28, which comprises the step of selecting an antibody whose antigen-binding activity at pH 6.7 to pH 10.0 is twice or higher that of the antigen-binding activity at pH 4.0 to pH 6.5.

30. A method of screening for an antigen-binding molecule, which comprises the steps of:
(a) binding an antigen-binding molecule to an antigen under a condition of pH 6.7 to pH 10.0;
(b) placing the antigen-binding molecule that bound to the antigen of (a) under a condition of pH 4.0 to pH 6.5; and
(c) obtaining an antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5.

31. A method of screening for an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:
(a) binding an antigen-binding molecule to an antigen-immobilized column under the condition of a first pH;
(b) eluting the antigen-binding molecule that had bound to the column at the first pH from the column under the condition of a second pH; and
(c) collecting the eluted antigen-binding molecule.

32. A method of screening for an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:
(a) binding an antigen-binding molecule library to an antigen-immobilized column under the condition of a first pH;
(b) eluting the antigen-binding molecule from the column under the condition of a second pH;
(c) amplifying the gene encoding the eluted antigen-binding molecule; and
(d) obtaining the eluted antigen-binding molecule.

33. The screening method of claim 31 or 32, wherein the first pH is 6.7 to 10.0 and the second pH is 4.0 to 6.5.

34. The screening method of any one of claims 28 to 33, wherein at least one or more amino acids of the antigen-binding molecule has been substituted with histidine, or at least one histidine has been inserted into the antigen-binding molecule.

35. The screening method of any one of claims 28 to 33, for obtaining an antigen-binding molecule that is superior in retention in the plasma.

36. The screening method of any one of claims 28 to 33, for obtaining an antigen-binding molecule that is capable of binding to an antigen two or more times.

37. The screening method of any one of claims 28 to 33, for obtaining an antigen-binding molecule that is capable of binding to more antigens as compared to the number of its antigen-binding sites.

38. The screening method of any one of claims 28 to 33, for obtaining an antigen-binding molecule that dissociates an extracellularly-bound antigen within a cell.

39. The screening method of any one of claims 28 to 33, for obtaining an antigen-binding molecule that is bound to an antigen and internalized into a cell, and released to the outside of the cell in an antigen-free form.

40. The screening method of any one of claims 28 to 33, for obtaining an antigen-binding molecule that has increased ability to eliminate an antigen in plasma.

41. The screening method of any one of claims 28 to 40, wherein the antigen-binding molecule is used as a pharmaceutical composition.

42. The screening method of any one of claims 28 to 41, wherein the antigen-binding molecule is an antibody.

43. A method for producing an antigen-binding molecule, which comprises the steps of:
(a) determining the antigen-binding activity of an antigen-binding molecule at pH 6.7 to pH 10.0;
(b) determining the antigen-binding activity of the antigen-binding molecule at pH 4.0 to pH 6.5;
(c) selecting the antigen-binding molecule whose antigen-binding activity at pH 6.7 to pH 10.0 is greater than that at pH 4.0 to pH 6.5;
(d) obtaining the gene encoding the antigen-binding molecule selected in (c); and
(e) producing the antigen-binding molecule using the gene obtained in (d).

44. A method for producing an antigen-binding molecule, which comprises the steps of:
(a) binding an antigen-binding molecule to an antigen under a condition of pH 6.7 to pH 10.0;

(b) allowing the antigen-binding molecule bound to the antigen of (a) to stand under a condition of pH 4.0 to pH 6.5;
(c) collecting the antigen-binding molecule that dissociated under the condition of pH 4.0 to pH 6.5;
(d) obtaining the gene encoding the antigen-binding molecule obtained in (c); and
(e) producing the antigen-binding molecule using the gene obtained in (d).

45. A method for producing an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:
(a) binding an antigen-binding molecule to an antigen-immobilized column under the first pH condition;
(b) eluting the antigen-binding molecule, which is bound to the column at the first pH, from the column under a second pH condition;
(c) collecting the eluted antigen-binding molecule;
(d) obtaining the gene encoding the antigen-binding molecule obtained in (c); and
(e) producing the antigen-binding molecule using the gene obtained in (d).

46. A method for producing an antigen-binding molecule whose binding activity at a first pH is greater than that at a second pH, which comprises the steps of:
(a) binding an antigen-binding molecule library to an antigen-immobilized column under the first pH condition;
(b) eluting the antigen-binding molecule from the column under the second pH condition;
(c) amplifying the gene encoding the eluted antigen-binding molecule;
(d) collecting the eluted antigen-binding molecule;
(e) obtaining the gene encoding the antigen-binding molecule collected in (d); and
(f) producing the antigen-binding molecule using the gene obtained in (e).

47. The production method of claim 45 or 46, wherein the first pH is 6.7 to 10.0 and the second pH is 4.0 to 6.5.

48. The production method of any one of claims 43 to 47, which further comprises the step of substituting at least one amino acid of the antigen-binding molecule with histidine, or inserting at least one histidine into the antigen-binding molecule.

49. The production method of any one of claims 43 to 48, wherein the antigen-binding molecule is an antibody.

50. A pharmaceutical composition comprising an antigen-binding molecule produced by the production method of any one of claims 43 to 49.

SEQUENCE LISTING

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<120> ANTIGEN-BINDING MOLECULE CAPABLE OF BINDING TO TWO OR MORE ANTIGEN MOLECULES REPEATEDLY

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<141> 2009-04-10

<150> JP 2008-104147
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<150> JP 2008-247713
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35 40 45

Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu
50 55 60

Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser
65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys
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35 40 45

Tyr Tyr Gly Ser Glu Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala
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35 40 45

Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu
50 55 60

Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser
65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys
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Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly
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Thr Leu Val Thr Val Ser Ser

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20 25 30

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35 40 45

Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr His Tyr Asn Pro His Leu
50 55 60

Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser
65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys
85 90 95

Ala Arg Val Leu Ala Arg Ile Thr Ala Met Asp His Trp Gly Glu Gly
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35 40 45

Ile Gly His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu
50 55 60

Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser

65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys
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Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly
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35 40 45

Tyr Tyr Gly Ser His Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala
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Tyr Tyr Gly Ser His Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

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35 40 45

Tyr Tyr Gly Ser His Leu His His Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala
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Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly His Arg Leu Pro Tyr
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His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp
35 40 45

Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr Thr Tyr Asn Pro Ser Leu
50 55 60

Lys Ser Arg Val Thr Met Leu Arg Asp Thr Ser Lys Asn Gln Phe Ser
65 70 75 80

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

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Gln Gly
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Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu

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Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn
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Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu

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50 55 60

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

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100 105 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
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Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
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Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
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Phe Asn Arg Gly Glu Cys
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20 25 30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
35 40 45

Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu
50 55 60

Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser
65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys
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1               5                  10                  15

Asp Ser Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ser Ser Tyr
            20                  25                  30

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

Tyr Tyr Gly Ser Glu Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala
65                  70                  75                  80

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

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Glu
            100                 105
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<210> 13
<211> 1404
<212> DNA
<213> Artificial

<220>
<223> An artificially synthesized nucleotide sequence

<400> 13
atgggatgga gctgtatcat cctcttcttg gtagcaacag ctacaggtgt ccactcccag 60

gtccaactgc aggagagcgg tccaggtctt gtgaaacctа gcgagaccct gagcctgacc 120

tgcgccgtgt ctggctactc aattagcgac gatcatgcct ggagctgggt tcgccagcca 180

cctggagaag gtcttgagtg gattggatac attagttata gtggaatcac aaactataat 240

ccatctctca aaggcagagt gacaatatcg agagacacca gcaagaacca gttcagcctg 300

aaactcagca gcgtgacagc cgccgacacc gcggcttatt attgtgcaag atccctagct 360

cggactacgg ctatggacta ctggggtgaa ggcaccctcg tcacagtctc ctcagcctcc 420

accaagggcc catcggtctt ccccctggca ccctcctcca agagcacctc tgggggcaca 480

gcggccctgg gctgcctggt caaggactac ttccccgaac cggtgacggt gtcgtggaac 540

tcaggcgccc tgaccagcgg cgtgcacacc ttcccggctg tcctacagtc ctcaggactc 600

tactccctca gcagcgtggt gaccgtgccc tccagcagct tgggcaccca gacctacatc 660

tgcaacgtga atcacaagcc cagcaacacc aaggtggaca agaaagttga gcccaaatct 720

tgtgacaaaa ctcacacatg cccaccgtgc ccagcacctg aactcctggg gggaccgtca 780

gtcttcctct tccccccaaa acccaaggac accctcatga tctcccggac ccctgaggtc 840

acatgcgtgg tggtggacgt gagccacgaa gaccctgagg tcaagttcaa ctggtacgtg 900

gacggcgtgg aggtgcataa tgccaagaca aagccgcggg aggagcagta caacagcacg 960

taccgtgtgg tcagcgtcct caccgtcctg caccaggact ggctgaatgg caaggagtac 1020

aagtgcaagg tctccaacaa agccctccca gcccccatcg agaaaaccat ctccaaagcc 1080

aaagggcagc cccgagaacc acaggtgtac accctgcccc catcccggga tgagctgacc 1140

aagaaccagg tcagcctgac ctgcctggtc aaaggcttct atcccagcga catcgccgtg 1200

gagtgggaga gcaatgggca gccggagaac aactacaaga ccacgcctcc cgtgctggac 1260

tccgacggct ccttcttcct ctacagcaag ctcaccgtgg acaagagcag gtggcagcag 1320

gggaacgtct tctcatgctc cgtgatgcat gaggctctgc acaaccacta cacgcagaag 1380

agcctctccc tgtctccggg taaa 1404

<210> 14
<211> 321
<212> DNA
<213> Artificial

<220>
<223> An artificially synthesized nucleotide sequence

<400> 14
gacatccaga tgacccagag cccaagcagc ctgagcgcca gcgtgggtga cagcgtgacc 60

atcacctgtc aagccagcca ggacatcagc agttacctga attggtacca gcagaagcca 120

ggaaaggctc cagagctgct gatctactac ggctccgaac tgcactctgg tgtgccaagc 180

agattcagcg gtagcggtag cggtaccgac ttcaccttca ccatcagcag cctcgaggca 240

gaggacgccg ctacctacta ctgcgggcag ggtaaccggc ttccatacac gttcggccaa 300

gggaccaagg tggaaatcga a 321

<210> 15
<211> 15
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 15

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10 15

<210> 16
<211> 24
<212> DNA
<213> Artificial

<220>
<223> An artificially syntheisized primer sequence

<400> 16
ttcccaccag cctgtccgcc tctg 24

<210> 17

<211> 24
<212> DNA
<213> Artificial

<220>
<223> An artificially syntheisized primer sequence

<400> 17
cgtgaagcca aaggccccac tccc 24

<210> 18
<211> 32
<212> DNA
<213> Artificial

<220>
<223> An artificially syntheisized primer sequence

<400> 18
tagaattcca ccatgctggc cgtcggctgc gc 32

<210> 19
<211> 57
<212> DNA
<213> Artificial

<220>
<223> An artificially syntheisized primer sequence

<400> 19
attgcggccg cttatcagtg gtgatgatga tgatgtggta ccgaagaaga atcttgc 57

<210> 20

<211> 1131
<212> DNA
<213> Artificial

<220>
<223> An artificially synthesized nucleotide sequence

<400> 20
gaattccacc atgctggccg tcggctgcgc gctgctggct gccctgctgg ccgcgccggg 60

ggcggcgctg gccccggggg gctgccctgc acaggaggtg gcgagaggtg tgctgaccag 120

tctgccagga gacagcgtga ctctgacctg cccagcggga gagccggaag acaatgccac 180

tgttcactgg gttctcagga agccagctgt aggctcccac ctcagcagat gggctggcgt 240

gggaaggagg ctgctgctga ggtcggtgca gctccatgac tctggaaact attcatgcta 300

ccgggccggc cgcccggctg gaactgtgca cttgctggtg gatgttcccc ccgaggagcc 360

ccagctctcc tgcttccgga agagcccccc cagcaacgtt gtttgtgagt ggggtcctcg 420

gagcacccca tctccgacga ccaaggctgt gctgttggtg aggaagtttc agaacagtcc 480

ggccgaagac ttccaggagc cgtgccagta ttcccaggag tcccagaagt tctcctgcca 540

gttggcagtc ccggagggag acagctcttt ctacatagtg tccatgtgcg tcgccagtag 600

tgtcgggagc aagctcagca aaactcagac ctttcagggt tgtggaatct tgcagcctga 660

tccgcctgcc aacatcacag tcactgccgt ggccagaaac ccccgctggc tcagtgtcac 720

ctggcaagac ccccactcct ggaactcatc tttctacaga ctacggtttg agctcagata 780

tcgagctgaa cggtcaaaga cattcacaac atggatggtc aaggacctcc agcatcactg 840

tgtcatccac gacgcctgga gcggcctgag gcacgtggtg cagcttcgtg cccaggagga 900

gttcgggcaa ggcgagtgga gcgagtggag cccggaggcc atgggcacgc cttggacaga 960

atccaggagt cctccagctg agaacgaggt gtccaccccc acgcaggcac ctactactaa 1020

taaagatgat gataatattc tctccagaga ttctgcaaat gcgacaagcc tcccagtgca 1080

agattcttct tcggtaccac atcatcatca tcaccactga taagcggccg c 1131

<210> 21
<211> 449
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 21

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser His Asp
20 25 30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
35 40 45

Ile Gly His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu
50 55 60

Gln Asp Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser
65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys
85 90 95

Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly
100 105 110

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

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu
130 135 140

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
210 215 220

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
225 230 235 240

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

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

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

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

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

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

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

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

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

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

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

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

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

Lys

<210> 22
<211> 449
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 22

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser His Asp
            20                  25                  30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
        35                  40                  45

Ile Gly His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu
    50                  55                  60

Gln Asp Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser
65                  70                  75                  80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly
            100                 105                 110

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

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu
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130 135 140

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
210 215 220

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
225 230 235 240

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

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

Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn

275 280 285

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

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

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

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

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

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

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

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

Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu

420 425 430

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

Lys

<210> 23
<211> 449
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 23

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp
20 25 30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
35 40 45

Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu
50 55 60

Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65 70 75 80

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

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly
100 105 110

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

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu
130 135 140

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
210 215 220

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
225 230 235 240

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

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

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

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

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

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

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

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

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

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

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

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

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

Lys

<210> 24
<211> 449
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 24

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp
20 25 30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
35 40 45

Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Thr Leu
50 55 60

Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65 70 75 80

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

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly
100 105 110

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

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu

130 135 140

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
210 215 220

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
225 230 235 240

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

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

Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn

275 280 285

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

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

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

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

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

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

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

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

Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu

420 425 430

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

Lys

<210> 25
<211> 214
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 25

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

Asp Ser Val Thr Ile Thr Cys Gln Ala Ser Arg Asp Ile Ser Ser His
20 25 30

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

Tyr Tyr Gly Ser His Leu Leu Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala
65 70 75 80

Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly Asn Arg Leu Pro Tyr
85 90 95

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

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

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

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

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

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

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

Phe Asn Arg Gly Glu Cys
210

<210> 26
<211> 214
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 26

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

Asp Ser Val Thr Ile Thr Cys Gln Ala Ser Arg Asp Ile Ser Ser His
20 25 30

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

Tyr Tyr Gly Ser His Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala
65 70 75 80

Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly Asn Arg Leu Pro Tyr

85 90 95

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

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

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

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

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

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

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

Phe Asn Arg Gly Glu Cys
210

<210> 27

<211> 214
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 27

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

Asp Ser Val Thr Ile Thr Cys Gln Ala Ser Thr Asp Ile Ser Ser His
20 25 30

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

Tyr Tyr Gly Ser His Leu Leu Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Glu Ala
65 70 75 80

Glu Asp Ala Ala Thr Tyr Tyr Cys Gly Gln Gly Asn Arg Leu Pro Tyr
85 90 95

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

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

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

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

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

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

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

Phe Asn Arg Gly Glu Cys
210

<210> 28
<211> 326
<212> PRT
<213> Homo sapiens

<400> 28

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Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg
1               5                  10                  15

Ser Thr Ser Glu Ser Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
            20                  25                  30

Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
        35                  40                  45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
    50                  55                  60

Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn Phe Gly Thr Gln Thr
65                  70                  75                  80

Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys
                85                  90                  95

Thr Val Glu Arg Lys Cys Cys Val Glu Cys Pro Pro Cys Pro Ala Pro
            100                 105                 110

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

Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp
    130                 135                 140
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Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly
145 150 155 160

Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn
165 170 175

Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp
180 185 190

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

Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu
210 215 220

Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn
225 230 235 240

Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile
245 250 255

Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr
260 265 270

Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys
275 280 285

Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys
290 295 300

Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu
305 310 315 320

Ser Leu Ser Pro Gly Lys
325

<210> 29
<211> 324
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 29

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg
1 5 10 15

Ser Thr Ser Glu Ser Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
50 55 60

Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn Phe Gly Thr Gln Thr
65 70 75 80

Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys
85 90 95

Thr Val Glu Arg Lys Cys Cys Val Glu Cys Pro Pro Cys Pro Ala Pro
100 105 110

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

Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp
130 135 140

Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly
145 150 155 160

Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn
165 170 175

Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp
180 185 190

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

Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu
210 215 220

Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn
225 230 235 240

Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile
245 250 255

Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr
260 265 270

Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys
275 280 285

Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys
290 295 300

Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu
305 310 315 320

Ser Leu Ser Pro

<210> 30
<211> 324

<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 30

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Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
1               5                  10                  15

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

Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
        35                  40                  45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
    50                  55                  60

Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn Phe Gly Thr Gln Thr
65                  70                  75                  80

Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys
                85                  90                  95

Thr Val Glu Arg Lys Ser Cys Val Glu Cys Pro Pro Cys Pro Ala Pro
            100                 105                 110
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Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp
115 120 125

Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp
130 135 140

Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly
145 150 155 160

Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn
165 170 175

Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp
180 185 190

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

Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu
210 215 220

Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn
225 230 235 240

Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile
245 250 255

Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr
260 265 270

Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys
275 280 285

Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys
290 295 300

Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu
305 310 315 320

Ser Leu Ser Pro

<210> 31
<211> 326
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 31

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg
1 5 10 15

Ser Thr Ser Glu Ser Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
50 55 60

Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn Phe Gly Thr Gln Thr
65 70 75 80

Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys
85 90 95

Thr Val Glu Arg Lys Cys Cys Val Glu Cys Pro Pro Cys Pro Ala Pro
100 105 110

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

Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp
130 135 140

Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly
145 150 155 160

Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn
165 170 175

Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp
180 185 190

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

Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu
210 215 220

Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn
225 230 235 240

Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile
245 250 255

Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr
260 265 270

Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys
275 280 285

Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys
290 295 300

Ser Val Met His Glu Ala Leu His Ala His Tyr Thr Gln Lys Ser Leu
305 310 315 320

Ser Leu Ser Pro Gly Lys
325

<210> 32
<211> 324
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 32

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
1 5 10 15

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

Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
50 55 60

Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn Phe Gly Thr Gln Thr
65 70 75 80

Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys
85 90 95

Thr Val Glu Arg Lys Ser Cys Val Glu Cys Pro Pro Cys Pro Ala Pro
100 105 110

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

Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp
130 135 140

Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly
145 150 155 160

Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn
165 170 175

Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp
180 185 190

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

Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu
210 215 220

Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn
225 230 235 240

Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile
245 250 255

Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr
260 265 270

Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys
275 280 285

Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys
290 295 300

Ser Val Met His Glu Ala Leu His Ala His Tyr Thr Gln Lys Ser Leu
305 310 315 320

Ser Leu Ser Pro

<210> 33
<211> 443
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 33

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp
            20                  25                  30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
        35                  40                  45

Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu
    50                  55                  60

Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

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

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly
            100                 105                 110

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

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

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Cys Cys Val Glu
210 215 220

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

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

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

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

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

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

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

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

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

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

Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly
385 390 395 400

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

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

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

<210> 34
<211> 443
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 34

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp
20 25 30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
35 40 45

Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu
50 55 60

Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65 70 75 80

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

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly
100 105 110

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

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu
130 135 140

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Ser Cys Val Glu
210 215 220

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

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

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

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

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

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

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

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

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

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

Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly
385 390 395 400

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

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

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

<210> 35
<211> 443
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 35

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp
20 25 30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
35 40 45

Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu
50 55 60

Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65 70 75 80

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

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly
100 105 110

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

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu
130 135 140

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Ser Cys Val Glu
210 215 220

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

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

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

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

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

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

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

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

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

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

Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly
385 390 395 400

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

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

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

<210> 36
<211> 445

<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 36

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser His Asp
20 25 30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
35 40 45

Ile Gly His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu
50 55 60

Gln Asp Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser
65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly
100 105 110

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

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

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Cys Cys Val Glu
210 215 220

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

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

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

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

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

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

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

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

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

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

Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly
385 390 395 400

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

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

His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
435 440 445

<210> 37
<211> 443
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 37

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser His Asp
20 25 30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
35 40 45

Ile Gly His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu
50 55 60

Gln Asp Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser
65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Phe Leu Ala Arg Ile Thr Ala His Asp His Trp Gly Glu Gly
100 105 110

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

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu
130 135 140

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Ser Cys Val Glu
210 215 220

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

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

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

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

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

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

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

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

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

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

Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly
385 390 395 400

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

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

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

<210> 38
<211> 445
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 38

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp
            20                  25                  30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
        35                  40                  45

Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Thr Leu
    50                  55                  60

Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

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

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly
            100                 105                 110

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

Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala Leu
    130                 135                 140
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Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
145 150 155 160

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

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

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

Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Cys Cys Val Glu
210 215 220

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

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

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

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

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

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

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

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

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

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

Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly
385 390 395 400

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

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

His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
435 440 445

<210> 39
<211> 443
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 39

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser His Asp
20 25 30

His Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
35 40 45

Ile Gly Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Thr Leu
50 55 60

Gln Gly Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65 70 75 80

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

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly
100 105 110

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

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu
130 135 140

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Ser Cys Val Glu
210 215 220

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

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

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

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

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

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

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

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

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

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

Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly
385 390 395 400

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

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

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

<210> 40
<211> 6
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 40

His Asp His Ala Trp Ser
1 5

<210> 41
<211> 16
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 41

His Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro His Leu Gln Asp
1 5 10 15

<210> 42
<211> 16
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 42

Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu Gln Gly
1 5 10 15

<210> 43
<211> 16
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 43

Phe Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Thr Leu Gln Gly
1 5 10 15

<210> 44
<211> 10
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 44

Phe Leu Ala Arg Ile Thr Ala His Asp His
1 5 10

<210> 45
<211> 10
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 45

Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr
1 5 10

<210> 46
<211> 30
<212> PRT
<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 46

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser
20 25 30

<210> 47
<211> 30
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 47

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly His Ser Ile Ser
20 25 30

<210> 48
<211> 14
<212> PRT
<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 48

Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp Ile Gly
1 5 10

<210> 49
<211> 32
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 49

Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser Leu Lys
1 5 10 15

Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys Ala Arg
20 25 30

<210> 50
<211> 32
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 50

Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser Leu Lys
1 5 10 15

Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys Ala Arg
20 25 30

<210> 51
<211> 32
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 51

Arg Val Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln
1 5 10 15

Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
20 25 30

<210> 52
<211> 11
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 52

Trp Gly Glu Gly Thr Leu Val Thr Val Ser Ser
1 5 10

<210> 53
<211> 11
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 53

Gln Ala Ser Arg Asp Ile Ser Ser His Leu Asn
1 5 10

<210> 54
<211> 11
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 54

Gln Ala Ser Thr Asp Ile Ser Ser His Leu Asn
1 5 10

<210> 55
<211> 7
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 55

Tyr Gly Ser His Leu Leu Ser
1 5

<210> 56
<211> 7
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 56

Tyr Gly Ser His Leu Glu Ser
1 5

<210> 57
<211> 9
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 57

Gly Gln Gly Asn Arg Leu Pro Tyr Thr
1 5

<210> 58
<211> 23
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 58

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

Asp Ser Val Thr Ile Thr Cys
20

<210> 59
<211> 15
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 59

Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Glu Leu Leu Ile Tyr
1 5 10 15

<210> 60
<211> 32

<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 60

Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
1 5 10 15

Phe Thr Ile Ser Ser Leu Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys
20 25 30

<210> 61
<211> 10
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 61

Phe Gly Gln Gly Thr Lys Val Glu Ile Glu
1 5 10

<210> 62
<211> 449
<212> PRT
<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 62

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

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Phe
20 25 30

Ala Met Ser Trp Phe Arg Gln Ser Pro Glu Lys Arg Leu Glu Trp Val
35 40 45

Ala Glu Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Thr Val
50 55 60

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

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

Ala Arg Gly Leu Trp Gly Tyr Tyr Ala Leu Asp Tyr Trp Gly Gln Gly
100 105 110

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

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu
130 135 140

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
210 215 220

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
225 230 235 240

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

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

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

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

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

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

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

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

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

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

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

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

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

Lys

<210> 63
<211> 213
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 63

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

Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
20 25 30

Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Arg Leu Leu Ile Tyr
35 40 45

Asp Thr Ser Asn Leu Ala Ser Gly Val Pro Val Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Arg Met Glu Ala Glu
65 70 75 80

Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Gly Tyr Pro Tyr Thr
85 90 95

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

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

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

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

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

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

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

Asn Arg Gly Glu Cys
210

<210> 64
<211> 449
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 64

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

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser His
20 25 30

Ala Met Ser Trp Phe Arg Gln Ser Pro Glu Lys Arg Leu Glu Trp Val
35 40 45

Ala Glu Ile Ser Ser Gly Gly Ser Tyr Thr Tyr His Pro His Thr Val
50 55 60

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

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

Ala Arg Gly Leu Trp Gly His Tyr Ala Leu Asp Tyr Trp Gly Gln Gly
100 105 110

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

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu
130 135 140

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
210 215 220

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
225 230 235 240

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

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

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

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

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

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

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

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

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

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

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

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

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

Lys

<210> 65
<211> 213
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 65

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

Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
20 25 30

Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Arg Leu Leu Ile Tyr
35 40 45

Asp Thr Ser Asn His Ala Ser Gly Val Pro Val Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Arg Met Glu Ala Glu
65 70 75 80

Asp Ala Ala Thr Tyr Tyr Cys Gln His Trp Ser Gly His Pro Tyr Thr
85 90 95

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

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

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

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

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

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

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

Asn Arg Gly Glu Cys
210

<210> 66
<211> 213
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 66

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

Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
20 25 30

Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Arg Leu Leu Ile Tyr
35 40 45

Asp Thr Ser His His Ala Ser Gly Val Pro Val Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Arg Met Glu Ala Glu
65 70 75 80

Asp Ala Ala Thr Tyr Tyr Cys Gln His Trp Ser Gly His Pro Tyr Thr
85 90 95

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

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

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

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

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

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

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

Asn Arg Gly Glu Cys
210

<210> 67
<211> 456
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 67

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

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

Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45

Gly Trp Ile Asn Pro Asn Ser Gly Gly Thr Asn Tyr Ala Gln Lys Phe
50 55 60

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

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

Ala Arg Glu Asp Val Val Pro Ala Ala Met Ser Phe Tyr Tyr Gly Met
100 105 110

Asp Val Trp Gly Arg Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr
115 120 125

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
130 135 140

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

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

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

Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys
195 200 205

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

Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
225 230 235 240

Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
245 250 255

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
260 265 270

Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
275 280 285

Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
290 295 300

Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
305 310 315 320

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
325 330 335

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

Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
355 360 365

Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
370 375 380

Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
385 390 395 400

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

Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
420 425 430

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

Leu Ser Leu Ser Pro Gly Lys Gly
450 455

<210> 68
<211> 214
<212> PRT
<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 68

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

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

Tyr Trp Tyr Gln Gln Glu Pro Gly Gln Ala Pro Val Leu Val Val Tyr
35 40 45

Asp Asp Thr Asp Arg Pro Ala Gly Ile Pro Glu Arg Phe Ser Gly Ala
50 55 60

Asn Ser Gly Asn Thr Ala Thr Leu Thr Ile Ser Arg Val Glu Ala Gly
65 70 75 80

Asp Glu Ala Asp Tyr Tyr Cys Gln Val Trp Asp Ser Ser Thr Asp His
85 90 95

Gly Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly Gln Pro Lys
100 105 110

Ala Asn Pro Thr Val Thr Leu Phe Pro Pro Ser Ser Glu Glu Leu Gln
115 120 125

Ala Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp Phe Tyr Pro Gly
130 135 140

Ala Val Thr Val Ala Trp Lys Ala Asp Gly Ser Pro Val Lys Ala Gly
145 150 155 160

Val Glu Thr Thr Lys Pro Ser Lys Gln Ser Asn Asn Lys Tyr Ala Ala
165 170 175

Ser Ser Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys Ser His Arg Ser
180 185 190

Tyr Ser Cys Gln Val Thr His Glu Gly Ser Thr Val Glu Lys Thr Val
195 200 205

Ala Pro Thr Glu Cys Ser
210

<210> 69
<211> 456
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 69

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

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

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

Gly Trp Ile Asn Pro Asn Ser Gly Gly Thr Asn Tyr Ala Gln Lys Phe
50 55 60

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

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

Ala Arg Glu Asp Val Val Pro Ala Ala Met Ser Phe Tyr Tyr Gly Met
100 105 110

Asp Val Trp Gly Arg Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr
115 120 125

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
130 135 140

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

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

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

Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys
195 200 205

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

Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
225 230 235 240

Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
245 250 255

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
260 265 270

Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
275 280 285

Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
290 295 300

Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
305 310 315 320

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
325 330 335

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

Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
355 360 365

Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
370 375 380

Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
385 390 395 400

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

Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
420 425 430

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

Leu Ser Leu Ser Pro Gly Lys Gly
450 455

<210> 70
<211> 449
<212> PRT
<213> Artificial

<220>
<223> An artificially synthesized peptide sequence

<400> 70

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

Thr Leu Ser Leu Thr Cys Ala Val Ser Gly Tyr Ser Ile Ser Asp Asp
20 25 30

Gln Ala Trp Ser Trp Val Arg Gln Pro Pro Gly Glu Gly Leu Glu Trp
35 40 45

Ile Gly Tyr Ile Ser Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu
50 55 60

Lys Gly Arg Val Thr Ile Ser Arg Asp Thr Ser Lys Asn Gln Phe Ser
65 70 75 80

Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Ala Tyr Tyr Cys
85 90 95

Ala Arg Ser Leu Ala Arg Thr Thr Ala Met Asp Tyr Trp Gly Glu Gly
100 105 110

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

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu
130 135 140

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

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

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

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

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
210 215 220

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
225 230 235 240

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

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

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

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

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

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

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

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

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

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

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

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

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

Lys