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(54) Title: METHOD FOR CONTROL OF BLOOD KINETICS OF ANTIBODY

(54) 発明の名称: 抗体の血中動態を制御する方法

(57) Abstract: It is found that the blood half-life of an IgG antibody, which is a polypeptide having a FcRn binding region, can be controlled by a method comprising modifying a variable region residue of the IgG antibody exposed on the surface to thereby control the surface charge of the IgG antibody. It is confirmed that the antibody whose blood half-life is controlled by the method retains its activity practically. The method is widely applicable to any polypeptide having a FcRn binding region such as an IgG antibody, which can be recycled through a salvage pathway, regardless of the type of a target antigen.

(57) 要約: FcRn 結合領域を含むポリペプチドである IgG 抗体の可変領域残基のうち、表面に露出している残基を改変し表面電荷をコントロールすることで IgG 抗体の血中半減期を制御できることを見出した。本発明の方法によって血中半減期が制御された抗体は、実際に活性を保持していることが確認された。本発明の方法は、標的抗原の種類によらず、IgG 抗体等の FcRn のサルベージ経路によりリサイクルされる FcRn 結合領域を含むポリペプチドに対して広く適用可能である。

METHODS FOR CONTROLLING BLOOD PHARMACOKINETICS OF ANTIBODIES

Technical Field

5 The present invention relates to methods for modifying antibodies to control pharmacokinetics of the antibodies in blood, pharmaceutical compositions comprising as an active ingredient an antibody whose pharmacokinetics in blood is controlled, and methods for producing the same.

Background Art

0 Since antibodies are highly stable in blood and have few adverse effects, they have drawn much attention as pharmaceuticals. There are a number of IgG type antibody pharmaceuticals available on the market and many such are currently under development (Non-patent Documents 1 and 2). Technologies for enhancing the effector function and such
5 have been developed to produce second-generation antibody pharmaceuticals. For example, known are technologies for enhancing antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) through amino acid substitutions in the Fc domain of an IgG antibody (Non-patent Document 3). In addition to such amino acid substitutions that result in enhancement of the effector function, there are reports on other amino acid substitutions
0 in the Fc domain, which prolong antibody half-life in blood (Non-patent Documents 4 and 5). Prolonging the antibody half-life in blood enables administration of antibody pharmaceuticals at reduced doses or at longer intervals and thereby results in providing low-cost, highly advantageous antibody pharmaceuticals. Specifically, the half-life in blood can be prolonged by modifying the Fc domain through amino acid substitutions that increase the affinity for the
25 neonatal Fc receptor known as the IgG salvage receptor. Alternatively, the half-life in blood can be prolonged by shuffling the constant region CH1, CH2, and CH3 domains (Non-patent Document 6). However, the amino acid sequences of constant regions of an IgG antibody are conserved in humans, and therefore it is best to keep the number of artificial amino acid substitutions in the constant regions to a minimum from the viewpoint of immunogenicity.

30 Reported techniques for substituting amino acids in IgG antibody variable regions include not only humanization (Non-patent Document 7) but also affinity maturation to enhance binding activity using amino acid substitutions in complementarity determining regions (CDRs) (Non-patent Document 8) and improvement of physicochemical stability through amino acid substitutions in frameworks (FRs) (Non-patent Document 9). Thus, unlike in constant regions,
35 amino acid substitution in variable regions is a general technique for improving function and

properties of an antibody. Since the CDR amino acid sequences of a humanized antibody are derived from a nonhuman animal, the risk of immunogenicity need not be regarded as a problem. Alternatively, if the FR sequence is the same as that of a human antibody publicly disclosed in the Kabat Database (<http://ftp.ebi.ac.uk/pub/databases/kabat/>) or the IMGT Database (<http://imgt.cines.fr/>), the immunogenicity risk is thought to be low. However, only the above-described amino acid substitutions in the constant region Fc are so far available as methods for improving the half-life of IgG antibodies in blood. There is no report on a method for improving the half-life of IgG antibody in blood using amino acid substitution in a variable region where the risk of immunogenicity is lower. The reason is that it was considered that half-life of IgG in blood strongly depends on its binding to the neonatal Fc receptor, the salvage receptor, and antigen-dependent IgG elimination (Non-patent Document 10), and variable regions have no significant influence on this half-life in blood. Meanwhile, the isoelectric point (pI) of IgG is decreased when IgG is anionized through succination (Non-patent Document 11), or the pI of IgG is increased when the antibody is cationized through modification using a polyamine (Non-patent Document 12). However, in both cases, the half-life in blood is shortened rather than prolonged. Thus, improvement of the half-life in blood has not been achieved by changing the pI through modification.

Meanwhile, the half-life of minibodies (low molecular weight antibodies) such as Fab and scFv is shorter than that of IgG, which is a whole antibody. Therefore, the half-life of minibodies in blood can be prolonged by modification using a polymer such as polyethylene glycol to reduce its renal excretion (Non-patent Document 13). In addition to the modification with a polymer, a shift of the isoelectric point (pI) has also been reported to modify the pharmacokinetics of minibodies in blood. For example, Non-patent Document 14 described that modification of anti-Tac Fab with an organic acid decreased its pI, resulting in improvement of AUC (Area Under Curve). In contrast, Non-patent Documents 15 and 16 described that modification of anti-Tac dsFv with an organic acid decreased its pI, resulting in reduction of AUC. Non-patent Document 17 demonstrated that the half-life ($t_{1/2}$) and AUC of anti-Tac-scFv toxin were reduced when its pI was decreased by modifying its variable regions through amino acid substitutions. Non-patent Document 18 describes that there was almost no change in the AUC of an scFv when its pI was decreased by adding amino acids to the C terminus. Thus, the AUC of a minibody may be increased or decreased when its pI is decreased by modification or amino acid substitution. Accordingly, the half-life of minibodies in blood cannot be exactly controlled as intended by shifting the pI.

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Disclosure of the Invention

[Problems to be Solved by the Invention]

25 The present invention was achieved in view of the above circumstances. An objective of the present invention is to provide methods for controlling the blood half-life of polypeptides comprising an FcRn-binding domain, such as IgG antibodies, by modifying the polypeptides through substitution of amino acid residues exposed on their surface; pharmaceutical compositions that comprise polypeptides comprising an FcRn-binding domain, whose half-life in blood is controlled by amino acid substitutions; and methods for producing the pharmaceutical
30 compositions.

[Means for Solving the Problems]

The present inventors conducted dedicated studies on methods for controlling the blood half-life of polypeptides comprising an FcRn-binding domain through amino acid substitutions. As a result, the present inventors developed methods for controlling the half-life of IgG
35 antibodies by controlling the surface charge through modification of residues exposed on the surface in the variable regions of the IgG antibodies, polypeptides comprising an FcRn-binding

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domain. Specifically, the present inventors discovered modification sites in variable regions to control the surface charge and the half-life of IgG antibodies in blood without influencing antibody structure and function. Furthermore, the present inventors confirmed that antibodies whose half-life in blood is controlled by the present invention actually retain their activity.

Regardless of the type of target antigen, the methods of the present invention are widely applicable to polypeptides comprising an FcRn-binding domain, such as IgGs, which are recycled via the FcRn salvage pathway, and whose major metabolic pathway is not renal excretion.

According to a first aspect, the present invention provides a method for producing a polypeptide comprising an FcRn-binding domain, whose pharmacokinetics in blood is controlled, wherein the method comprises:

(a) modifying a nucleic acid encoding a polypeptide comprising an FcRn-binding domain to change the charge of at least one amino acid residue that can be exposed on the surface of the polypeptide;

(b) culturing a host cell to express the nucleic acid;

(c) collecting the polypeptide comprising an FcRn-binding domain from the host cell culture; and

(d) comparing the blood pharmacokinetics of the polypeptide comprising an FcRn-binding domain to that of the polypeptide before the modification of the amino acid residue,

wherein the amino acid residue that can be exposed on the surface of the polypeptide comprising an FcRn-binding domain is located in a domain other than the FcRn-binding domain within the polypeptide.

According to a second aspect, the present invention provides a polypeptide comprising an FcRn-binding domain, which is produced by the method of the first aspect.

25 According to a third aspect, the present invention provides a method for controlling blood pharmacokinetics of a polypeptide comprising an FcRn-binding domain, which comprises changing the charge of at least one amino acid residue that can be exposed on the surface of the polypeptide and comparing the blood pharmacokinetics of the polypeptide comprising an FcRn-binding domain to that of the polypeptide before the modification of the
30 amino acid residue,

wherein the amino acid residue that can be exposed on the surface of the polypeptide comprising an FcRn-binding domain is located in a domain other than the FcRn-binding domain within the polypeptide.

According to a fourth aspect, the present invention provides a method for producing a humanized antibody comprising complementarity determining regions (CDRs) of a nonhuman animal, human-derived framework regions (FRs), and human constant regions, whose pharmacokinetics in blood is controlled, wherein the method comprises:

(a) modifying, at the time of humanization or after humanization, at least one amino acid residue that can be exposed on the surface in the CDRs or FRs so as to have a charge different from that of the corresponding amino acid residue in CDRs or FRs of the nonhuman antibody, and

(b) comparing the blood pharmacokinetics of the humanized antibody to that of the chimeric antibody whose variable regions are derived from an antibody of the nonhuman animal and whose constant regions are the same.

According to a fifth aspect, the present invention provides a method for producing an IgG antibody, whose pharmacokinetics in blood is controlled, wherein the method comprises:

(a) changing the charge of at least one amino acid residue selected from the amino acid residues at positions 10, 12, 23, 39, 43, and 105 according to the Kabat's numbering system in a heavy chain variable region, and

(b) comparing the blood pharmacokinetics of the IgG antibody to that of the IgG antibody before the modification of the amino acid residue.

The present invention relates to methods for controlling the blood half-life of polypeptides comprising an FcRn-binding domain, such as IgG antibodies, by modifying the polypeptides through substitution of amino acid residues exposed on their surface; pharmaceutical compositions that comprise polypeptides comprising an FcRn-binding domain, whose half-life in blood is controlled by amino acid substitutions; and methods for producing the pharmaceutical compositions.

More specifically, the present invention relates to the following:

[1] a method for producing a polypeptide comprising an FcRn-binding domain, whose pharmacokinetics in blood is controlled, wherein the method comprises:

(a) modifying a nucleic acid encoding a polypeptide comprising an FcRn-binding domain to change the charge of at least one amino acid residue that can be exposed on the surface of the polypeptide,

(b) culturing a host cell to express the nucleic acid, and

(c) collecting the polypeptide comprising an FcRn-binding domain from the host cell culture;

- [2] the method of [1], wherein the amino acid residue that can be exposed on the surface of the polypeptide comprising an FcRn-binding domain is located in a domain other than the FcRn-binding domain within the polypeptide;
- [3] the method of [2], wherein the FcRn-binding domain comprises an Fc or Fc-like domain;
- 5 [4] the method of [1], wherein the polypeptide comprising an FcRn-binding domain is an IgG antibody;
- [5] the method of [4], wherein the amino acid residue whose charge is changed in step (a) is an amino acid residue in a heavy chain or light chain variable region of the IgG antibody;
- [6] the method of [1], wherein the control of pharmacokinetics in blood is the control of any
0 one of the following parameters: half-life in blood, mean residence time in blood, or blood clearance;
- [7] the method of [1], wherein the change of charge of the amino acid residue in step (a) is achieved by an amino acid substitution;
- [8] a polypeptide comprising an FcRn-binding domain, which is produced by the method of
5 [1];

[9] a method for controlling blood pharmacokinetics of a polypeptide comprising an FcRn-binding domain, which comprises changing the charge of at least one amino acid residue that can be exposed on the surface of the polypeptide;

[10] the method of [9], wherein the amino acid residue that can be exposed on the surface of the polypeptide comprising an FcRn-binding domain is located in a domain other than the FcRn-binding domain within the polypeptide;

[11] the method of [10], wherein the FcRn-binding domain comprises an Fc or Fc-like domain;

[12] the method of [9], wherein the polypeptide comprising an FcRn-binding domain is an IgG antibody;

[13] the method of [12], wherein the amino acid residue whose charge is changed is an amino acid residue in a heavy chain or light chain variable region of the IgG antibody;

[14] the method of [9], wherein the control of pharmacokinetics in blood is the control of any one of the following parameters: half-life in blood, mean residence time in blood, or blood clearance;

[15] the method of [9], wherein the change of charge of the amino acid residue is achieved by an amino acid substitution;

[16] a polypeptide comprising an FcRn-binding domain, whose pharmacokinetics in blood is controlled by the method of [9];

[17] a humanized antibody comprising complementarity determining regions (CDRs) of a nonhuman animal, human-derived framework regions (FRs), and human constant regions, wherein at least one amino acid residue that can be exposed on the surface in the CDRs or FRs has a charge different from that of the corresponding amino acid residue in CDRs or FRs of the wild type, and wherein its pharmacokinetics in blood is controlled as compared to a chimeric antibody whose variable regions are derived from an antibody of the nonhuman animal and whose constant regions are the same;

[18] the humanized antibody of [17], wherein the human constant regions comprise a wild-type human Fc domain;

[19] a composition comprising the humanized antibody of [17] or [18] and a pharmaceutically acceptable carrier;

[20] a nucleic acid encoding a polypeptide constituting the humanized antibody of [17] or 18;

[21] a host cell comprising the nucleic acid of [20];

[22] a method for producing the humanized antibody of [17] or [18], which comprises culturing the host cell of [21] and collecting a polypeptide from the cell culture;

[23] an IgG antibody, in which the charge of at least one amino acid residue selected from the amino acid residues at positions 10, 12, 23, 39, 43, and 105 according to the Kabat's numbering

system in a heavy chain variable region is changed, and whose pharmacokinetics in blood is controlled as compared to before the modification of the amino acid residue;

[24] the IgG antibody of [23], wherein the modified amino acid residue is selected from the amino acid residues of group (a) or (b) below:

- 5 (a) glutamic acid (E) and aspartic acid (D), and
(b) lysine (K), arginine (R), and histidine (H);

[25] a composition comprising the IgG antibody of [23] or [24] and a pharmaceutically acceptable carrier;

[26] a nucleic acid encoding a polypeptide constituting the IgG antibody of [23] or [24];

- 10 [27] a host cell comprising the nucleic acid of [26]; and

[28] a method for producing the antibody of [23] or [24], which comprises culturing the host cell of [27] and collecting a polypeptide from the cell culture.

Brief Description of the Drawings

- 15 Fig. 1 is a graph showing the assessment of coagulation activity for a humanized bispecific antibody (humanized A69 (hA69a), humanized B26 (hB26-F123e4), and humanized BBA (hAL-F123j4)). The assessment result demonstrates that the coagulation activities are equivalent to or greater than those of chimeric bispecific antibodies.

- 20 Fig. 2 is a diagram showing antibody modeling for the combinations of humanized A69-H chain variable region (hA69a) and humanized BBA (hAL-F123j4), and humanized hB26-H chain variable region (hB26-F123e4) and humanized BBA (hAL-F123j4). The side chains of amino acids that can change the surface charge are shown emphasized. The numbering was done according to the Kabat database numbering system (Kabat EA *et al.*, (1991) Sequences of Proteins of Immunological Interest. NIH).

- 25 Fig. 3 is a photograph showing a result of isoelectric focusing analysis of ATF, hA69-PF, BiAb, hB26-PF, hA69-N97R, hA69-p18, hB26-F123e4, and hB26-p15.

- Fig. 4 shows a calibration curve of a pI marker and the pI of each sample determined from the curve, which were obtained from the isoelectric focusing analysis of ATF, hA69-PF, BiAb, hB26-PF, hA69-N97R, hA69-p18, hB26-F123e4, and hB26-p15. The diagrams
30 demonstrate that the surface charge varies depending on the amino acid sequences of variable regions and differences in the surface charge resulting from amino acid modifications shift the pI.

- Fig. 5 shows results of analyzing humanized A69 antibodies (hA69a and hA69-N97R; hA69-N97R, hA69-p18, and hA69-PF) with unmodified or modified variable regions for their
35 binding activity to the antigen Factor IXa. The results demonstrate that the modified antibodies

with shifted isoelectric points have a binding activity comparable to that of unmodified antibodies.

Fig. 6 is a graph showing a result of analyzing humanized B26 antibodies (hB26-F123e4 and hB26-p15) with unmodified or modified variable regions, respectively, for their binding activity to the antigen Factor X. The results demonstrate that the modified antibody with shifted isoelectric point has a binding activity comparable to that of the unmodified antibody.

Fig. 7 is a graph showing time courses of plasma concentrations of ATF, hA69-PF, BiAb, and hB26-PF.

Fig. 8 shows the correlation between pI and pharmacokinetic parameters, clearance (CL) and half-life ($T_{1/2}$), for ATF, hA69-PF, BiAb, hB26-PF, hA69-N97R, hA69-p18, hB26-F123e4, and hB26-p15.

Best Mode for Carrying Out the Invention

The present invention provides methods for controlling pharmacokinetics of polypeptides comprising an FcRn-binding domain in blood. In a preferred embodiment, the methods of the present invention comprise changing the charge of at least one amino acid residue that can be exposed on the surface of such polypeptides. Specifically, pharmacokinetics of these polypeptides in blood can be controlled by shifting their isoelectric point (pI) by changing the charge of their amino acid residues.

As described above, blood pharmacokinetics of minibodies such as scFvs and Fabs cannot necessarily be controlled by shifting pI. Renal excretion is known to be the major metabolic pathway of such minibodies. However, some of Non-patent Documents 19 to 22 describe that renal filtration efficiency in renal excretion is lower if protein charge is more negative, while others report that protein charge has no influence on renal filtration efficiency. In addition, some of Non-patent Documents 14 to 18 report that the half-life of a minibody in blood can be prolonged by decreasing its pI, while others report that it can be shortened by decreasing its pI. Proteins filtered through the kidney are reabsorbed by the proximal tubule. This reabsorption can become more suppressed when the charge of a protein is more negative. This suggests that the half-life of a minibody cannot be exactly controlled as intended by shifting the pI.

On the other hand, the major metabolic pathway of IgG antibodies is not renal excretion because their molecular weight is quite high. IgG antibodies with Fc are known to be recycled via the FcRn salvage pathway expressed in the endothelial cells of blood vessels and such, and thereby have a long half-life. IgG is assumed to be metabolized primarily in endothelial cells (Non-patent Document 23). Accordingly, it has been speculated that free IgG molecules are

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5 metabolized while IgG molecules non-specifically incorporated into endothelial cells are recycled via binding to FcRn. IgGs with decreased FcRn-binding activity have a shorter half-life in blood, while their half-life in blood can be prolonged by increasing their FcRn-binding activity (Non-patent Document 23). Accordingly, previous methods for controlling pharmacokinetics of IgGs in blood have been conducted by altering FcRn-binding activity through Fc modification. In contrast, Example 8 herein demonstrates that when IgGs share the same Fc domain, the IgG half-life correlates to pI showing a high correlation coefficient regardless of the type of target antigen, and that the half-life in blood of two types of antibodies against different antigens can actually be controlled by modifying the pIs of their variable regions without modifying Fc. The rate of nonspecific antibody uptake by endothelial cells is assumed to depend on the physicochemical Coulomb interaction between an IgG and a negatively charged cell surface. Therefore, it is thought that a decrease (an increase) in the pI of IgG reduces (enhances) the Coulomb interaction, and this is followed by a reduction (an increase) in nonspecific uptake by endothelial cells, leading to a decrease (an increase) in metabolism in these cells, and as a result, pharmacokinetics in blood can be controlled. Since the Coulomb interaction between negatively-charged cell surface of the endothelial cells is a physicochemical interaction, the interaction is assumed not to depend on the target antigen. Thus, the methods of the present invention for controlling pharmacokinetics in blood are widely applicable to polypeptides such as arbitrary IgGs comprising an FcRn-binding domain which are recycled via the FcRn salvage pathway and whose major metabolic pathway is not renal excretion, regardless of the type of antigen.

25 Thus, the polypeptides of the present invention comprising an FcRn-binding domain are not limited to IgG antibodies, but may be any protein that can bind (has binding activity or affinity) to an Fc receptor (FcRn). Thus, the polypeptides of the present invention comprising an FcRn-binding domain are not particularly limited; however, they are preferably proteins comprising an antibody Fc domain or Fc-like domain. The polypeptides of the present invention comprising an FcRn-binding domain include, for example, IgG antibodies. Furthermore, the polypeptides of the present invention comprising an FcRn-binding domain include modified forms of antibodies (proteins) as long as they can bind to FcRn. The most preferred examples of polypeptides of the present invention comprising an FcRn-binding domain are IgG antibodies.

30 When an IgG antibody is used as a polypeptide comprising an FcRn-binding domain in the present invention, it may be of any IgG subtype or a bispecific IgG antibody. Bispecific antibodies are antibodies specific to two different epitopes, and include antibodies that recognize different antigens and those that recognize different epitopes on a single antigen. On the other hand, when the antibody is a minibody such as scFv or Fab, whose major metabolic pathway is

renal excretion, its pharmacokinetics in blood cannot be controlled by shifting pI, as described above. The present invention is applicable to any type of antibody, as long as it is an Fc-binding protein whose major metabolic pathway is not renal excretion, for example, scFv-Fc, dAb-Fc, and Fc fusion proteins. Since renal excretion is not the major metabolic pathway of these molecules, their pharmacokinetics in blood can be controlled by shifting the pI using the methods of the present invention. Antibody molecules to which the present invention is applicable may be antibody-like molecules. Antibody-like molecules refer to molecules that exert their functions by binding to target molecules (Non-patent Document 30), and include, for example, DARPs, Affibodies, and Avimers.

Herein, the phrase "pharmacokinetics in blood is controlled" indicates that pharmacokinetics in blood is shifted in a desired direction when compared between before and after modification of a polypeptide comprising an FcRn-binding domain. Specifically, when the purpose is to prolong the half-life in blood, the control of pharmacokinetics in blood means prolongation of the half-life in blood. Alternatively, when the purpose is to reduce the half-life in blood, the control of pharmacokinetics in blood refers to reduction of the half-life in blood.

In the present invention, whether the blood pharmacokinetics of a polypeptide comprising an FcRn-binding domain is shifted in a desired direction, or whether the blood pharmacokinetics can be exactly controlled as intended, can be assessed by conducting appropriate kinetic tests, for example, tests using mice, rats, rabbits, dogs, monkeys or such.

More specifically, as used herein, "control of pharmacokinetics in blood" includes the control of any parameter such as half-life in blood, mean residence time in blood, or blood clearance (Pharmacokinetics: Enshu niyori Rikai (Understanding through Exercises), Nanzando). For example, the control of pharmacokinetics in blood can be assessed by appropriate noncompartmental analysis using *in vivo* kinetics analysis software, WinNonlin (Pharsight), according to the attached instruction manual.

Herein, the phrase "amino acids that can be exposed on the surface" generally means amino acids which constitute a polypeptide comprising an FcRn-binding domain and which are present on the polypeptide surface. The side chains of an amino acid that is present on the polypeptide surface can contact solvent molecules (typically, water molecules). However, not the whole side chain has to be in contact with solvent molecules. When even a portion of the side chains contacts solvent molecules, the amino acid is judged to be present on the polypeptide surface. Those skilled in art can prepare homology models for polypeptides and antibodies through homology modeling using commercially available software, and so on, and they can also select appropriate residues as surface amino acids using the models.

In the present invention, "amino acids that can be exposed on the surface" are not particularly limited; however, they are preferably outside an FcRn-binding domain of a

polypeptide comprising such a domain. An FcRn-binding domain includes, for example, Fc and Fc-like domains.

When a polypeptide of the present invention comprising an FcRn-binding domain is an IgG, amino acid residues whose charges are to be changed according to the present invention are preferably present within the heavy or light chain variable regions of the IgG antibody. Specifically, the variable regions include complementarity determining regions (CDRs) and framework regions (FRs).

Those skilled in art can select appropriate surface amino acids in antibody variable regions using homology models prepared by homology modeling or the like. Such surface amino acids include, for example, amino acids at H1, H3, H5, H8, H10, H12, H13, H15, H16, H19, H23, H25, H26, H39, H42, H43, H44, H46, H68, H71, H72, H73, H75, H76, H81, H82b, H83, H85, H86, H105, H108, H110, and H112 in an H chain FR region, but are not limited thereto in the present invention.

Likewise, those skilled in the art can also select surface amino acids in an H chain CDR region using homology models. For example, the amino acid at H97 is exposed on the surface in almost all antibodies. Surface amino acids in an L chain FR region include, for example, the amino acids at L1, L3, L7, L8, L9, L11, L12, L16, L17, L18, L20, L22, L38, L39, L41, L42, L43, L45, L46, L49, L57, L60, L63, L65, L66, L68, L69, L70, L74, L76, L77, L79, L80, L81, L85, L100, L103, L105, L106, L107, and L108, but are not limited thereto in the present invention.

Likewise, those skilled in art can also select surface amino acids in an L chain CDR region using homology models.

In the methods of the present invention, "modification" of an amino acid residue specifically refers to substitution of the original amino acid residue with a different amino acid residue, deletion of the original amino acid residue, addition of another amino acid residue, and so on. The modification preferably means substitution of the original amino acid residue with a different amino acid residue. Specifically, as used herein, "modification of the charge of an amino acid residue" preferably includes amino acid substitutions.

When a polypeptide of the present invention comprising an FcRn-binding domain is an IgG antibody, the above-described "changing the charge of an amino acid residue" includes, for example, changing the charge of at least one amino acid residue selected from the group consisting of amino acid residues at positions 10, 12, 23, 39, 43, and 105 according to the Kabat's numbering system in a heavy chain of the IgG antibody. Of the amino acid residues at the positions indicated above, amino acid residues other than the amino acid residues whose charges have been modified need not be modified as long as the pharmacokinetics in blood have been controlled as intended. The amino acids can be modified to have a charge of the same type as that of modified amino acid residues or not to have any charge.

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5 Amino acids are known to include charged amino acids. Generally known amino acids with a positive charge (positively-charged amino acids) include lysine (K), arginine (R), and histidine (H). Known amino acids with a negative charge (negatively-charged amino acids) include aspartic acid (D) and glutamic acid (E). Amino acids other than these are known as uncharged amino acids.

Preferably, the above-described "modified amino acid residues" are appropriately selected from amino acid residues of either group (a) or (b) indicated below; however, the modified amino acids are not particularly limited thereto.

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

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

When an original (unmodified) amino acid residue already has a charge, modification of the amino acid into an uncharged amino acid is also a preferred embodiment of the present invention. Specifically, the modification in the present invention includes:

(1) substitution of a charged amino acid with an uncharged amino acid;

(2) substitution of a charged amino acid with an amino acid carrying a charge opposite from the original amino acid; and

(3) substitution of an uncharged amino acid with a charged amino acid.

In the methods of the present invention, preferably, amino acid residues in polypeptides comprising an FcRn-binding domain are modified to shift the isoelectric point (pI). When the number of amino acid residues to be introduced through the modification is two or more, a few of them may be uncharged amino residues.

The number of amino acid residues to be modified in the methods of the present invention is not particularly limited. However, when an antibody variable region is modified for instance, only a minimum number of amino acid residues required to achieve the blood pharmacokinetics controlled as intended are preferably modified so as not to reduce the antigen-binding activity and not to increase the immunogenicity.

The amino acid sequence after modification is preferably a human sequence so as not to increase the immunogenicity; however, the present invention is not limited thereto.

Furthermore, mutations may be introduced at sites other than those where modifications have been made for shifting the isoelectric point, so that the respective FRs (FR1, FR2, FR3, and FR4) after modification are human sequences. Such a method for substituting each FR with a human sequence has been reported in a non-patent document (Ono K *et al.*, Mol Immunol. 1999 Apr; 36(6): 387-395). Alternatively, to shift the isoelectric point, each FR may be modified into another human FR with a different isoelectric point (for example, FR3 may be substituted by another human FR with a lower isoelectric point). Such a humanization method has been reported in a non-patent document (Dall'Acqua WF, Methods. 2005 May; 36(1): 43-60).

Even when pharmacokinetics in blood cannot be controlled as intended by modifying only a small number of surface charges, a desired polypeptide comprising an FcRn-binding domain, whose pharmacokinetics in blood is controlled, can be obtained by repeating surface charge modifications and assessment of pharmacokinetics in blood.

5 In rhesus monkeys, Non-patent Document 24 reports on a comparison of blood pharmacokinetics between chimeric EP5C7.g4, a chimeric antibody (IgG4), and HuEP5C7.g4, a humanized antibody (IgG4), both of which are derived from an anti-E, P-Selectin antibody, which found that the pharmacokinetics were comparable to each other. Non-patent Document 25 describes a comparison of blood pharmacokinetics in cynomolgus monkeys between ch5d8, a
10 chimeric antibody, and Hu5c8, a humanized antibody, both of which are derived from an anti-CD154 antibody, which found the pharmacokinetics to be comparable to each other. Non-patent Document 26 demonstrates that blood pharmacokinetics in mice of cCC49, a chimeric antibody, was comparable to that of HuCC49, a humanized antibody. Furthermore, Non-patent Documents 27 and 28 reports that pharmacokinetics and distribution in blood of
15 mouse antibodies were comparable to those of humanized antibodies when assessed using mice. Since both mouse Fcs and human Fcs are reactive to mouse FcRn, the above findings suggest that pharmacokinetics and distribution in blood of chimeric antibodies are comparable to those of the humanized antibodies described above. As shown by these examples, pharmacokinetics of chimeric antibodies in blood are comparable to that of humanized antibodies. Specifically,
20 when an antibody is humanized by a known method such as that described in Non-patent Document 7, its pharmacokinetics in blood is comparable to that of a chimeric antibody. Thus, humanized antibodies whose pharmacokinetics in blood is controlled cannot be produced by known methods.

Humanized antibodies whose pharmacokinetics in blood is controlled (specifically, the
25 half-life in blood is prolonged or pharmacokinetics in blood is reduced) as compared to chimeric antibodies can be produced by shifting their pIs through modification of surface amino acids at the time of humanization of chimeric antibodies, using the methods discovered by the present invention. The modification of surface amino acids to control pharmacokinetics in blood may be made at the time of humanization or after humanization.

30 Non-patent Document 29 describes that pharmacokinetics in blood of three types of humanized antibodies, trastuzumab, bevacizumab, and pertuzumab, obtained through humanization using the same FR sequence of a human antibody, was nearly the same. Specifically, pharmacokinetics in blood is nearly the same when humanization is performed using the same FR sequence. The blood concentration can be controlled only when the pIs of
35 antibodies are shifted by modifying surface amino acids using the methods discovered by the present invention, in addition to the above-described humanization process.

Furthermore, human antibodies whose pharmacokinetics in blood is controlled (specifically, the half-life in blood is prolonged or pharmacokinetics in blood is reduced) as compared to the original human antibodies can be produced by shifting the pIs of human antibodies prepared from human antibody libraries or mice producing human antibodies and such, through modification of surface amino acids.

The "antibodies" of the present invention include antibodies obtained by further introducing amino acid substitutions, deletions, additions and/or insertions and such into the amino acid sequences of antibodies that have been already modified to change the charges of their amino acid residues as described above. The antibodies of the present invention also include antibodies obtained by further changing the charge of the amino acid residues in antibodies whose amino acid sequences have been already modified by amino acid substitutions, deletions, additions and/or insertions, chimerization, humanization or such.

Amino acid modifications such as amino acid substitutions, deletions, additions and/or insertions, and chimerization and humanization, can be achieved by methods known to those skilled in the art. Likewise, the amino acid sequences of antibody constant and variable regions that are used to produce antibodies of the present invention as recombinant antibodies may also be modified by amino acid substitutions, deletions, additions and/or insertions, or chimerization, humanization or such.

The antibodies of the present invention may be antibodies derived from any animal such as a mouse, human, rat, rabbit, goat, or camel. Furthermore, the antibodies may be modified antibodies, for example, chimeric antibodies and in particular, humanized antibodies that comprise amino acid substitutions in their sequence. The antibodies also include antibody modification products linked to various molecules.

"Chimeric antibodies" are antibodies prepared by combining sequences derived from different animals. Chimeric antibodies include, for example, antibodies comprising heavy and light chain variable (V) regions from a mouse antibody and heavy and light chain constant (C) regions from a human antibody. Chimeric antibodies can be prepared by known methods, for example, by the following procedure: a DNA encoding an antibody V region is ligated with a DNA encoding a human antibody C region; the resulting ligation product is inserted into an expression vector; and the construct can be introduced into a host to produce a chimeric antibody.

"Humanized antibodies" are also referred to as reshaped human antibodies, and can be obtained by substituting the complementarity determining regions (CDRs) of a human antibody for the CDRs of an antibody derived from a nonhuman mammal, for example, a mouse.

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 techniques for the above procedure are also known (see European Patent Application Publication No. EP 125023, and WO 96/02576). For example, the CDRs of a mouse antibody are determined by known methods, and a DNA is prepared so that it encodes an antibody in which the CDRs are linked to the framework regions (FRs) of a human antibody. A humanized antibody can then be produced by a system using a conventional expression vector. Such DNAs can be synthesized by PCR using as primers several oligonucleotides designed to comprise portions that overlap the ends of both the CDR and FR regions (see the method described in WO 98/13388). Human antibody FRs linked via CDRs are selected such that the CDRs can form a suitable antigen binding site. If required, amino acids in the FRs of an antibody variable region may be modified so that the CDRs of a reshaped human antibody can form a suitable antigen binding site (Sato, K. *et al.*, Cancer Res. (1993) 53: 851-856). Modifiable amino acid residues in the FRs include portions that directly bind to an antigen via noncovalent bonds (Amit *et al.*, Science (1986) 233: 747-53), portions that have an impact or effect on the CDR structure (Chothia *et al.*, J. Mol. Biol. (1987) 196: 901-17), and portions involved in the interaction between VH and VL (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, C γ 1, C γ 2, C γ 3, and C γ 4 can be used for the H chains, and C κ and C λ can be used for the L chains. Meanwhile, the human antibody C regions may be modified as required to improve antibody or production stability. A chimeric antibody of the present invention preferably comprises a variable region of an antibody derived from a nonhuman mammal and a constant region derived from a human antibody. A humanized antibody of the present invention preferably comprises CDRs of an antibody derived from a nonhuman mammal and FRs and C regions derived from a human antibody. Constant regions of human antibodies comprise amino acid sequences specific to each antibody isotype, for example, IgG (IgG1, IgG2, IgG3, and IgG4), IgM, IgA, IgD, and IgE. The constant regions used to prepare the humanized antibodies of the present invention may be the constant regions of antibodies of any isotype. A constant region of a human IgG is preferably used, but the constant regions are not limited thereto. FRs derived from a human antibody, which are used to prepare humanized antibodies, are not particularly limited, and thus may be derived from an antibody of any isotype.

The variable and constant regions of chimeric or humanized antibodies of the present invention may be modified by deletions, substitutions, insertions, and/or additions, as long as the antibodies exhibit the same binding specificity as that of the original antibodies.

Chimeric and humanized antibodies using human-derived sequences are expected to be useful when administered to humans for therapeutic purposes or such, since their immunogenicity in the human body has been reduced.

Known sequences can be used as genes encoding the H chain or L chain of antibodies before introduction of mutations by methods of the present invention (herein, they may be simply referred to as "antibodies of the present invention"), or the genes can be obtained by methods known to those skilled in the art. For example, they may be obtained from an antibody library, or by cloning genes encoding the antibodies from hybridomas producing monoclonal antibodies.

Regarding antibody libraries, many antibody libraries are already well known, and since methods for producing antibody libraries are known, those skilled in the art can appropriately obtain antibody libraries. For example, with regard to 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: 3245-60; Vaughan *et al.*, Nature Biotechnology 1996, 14: 309-14; and Japanese Patent Kohyo Publication No. H10-504970 (unexamined Japanese national phase publication corresponding to a non-Japanese international publication). In addition, known methods such as methods that use eukaryotic cells as libraries (WO95/15393) and ribosome display methods, may be used. Furthermore, techniques 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 are 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 WO92/01047, WO92/20791, WO93/06213, WO93/11236, WO93/19172, WO95/01438, and WO95/15388.

As for methods for obtaining genes encoding antibodies from hybridomas, known techniques may be used, involving 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 immune cells thus obtained with known parent cells by ordinary cell fusion methods, screening monoclonal antibody producing cells (hybridomas) by ordinary screening methods, synthesizing cDNAs of antibody variable regions (V regions) from mRNAs of the obtained hybridomas using a reverse transcriptase, and linking them to DNAs encoding the desired antibody constant regions (C regions).

More specifically, without being particularly limited to the following examples, sensitizing antigens for obtaining the above-mentioned antibody genes encoding the H chains and L chains include both complete antigens with immunogenicity and incomplete antigens comprising haptens and such that do not show immunogenicity. For example, full-length

proteins and partial peptides of proteins of interest can be used. In addition, it is known that substances composed of polysaccharides, nucleic acids, lipids and such may become antigens. Thus, there are no particular limitations on antigens of the antibodies of the present invention. Antigens can be prepared by methods known to those skilled in the art, and they can be prepared, for example, by the methods using baculoviruses (for example, see WO98/46777). Hybridomas can be produced, for example, by the method of Milstein *et al.* (G. Kohler and C. Milstein, Methods Enzymol. 1981, 73: 3-46). When the immunogenicity of an antigen is low, it can be linked to a macromolecule that has immunogenicity, such as albumin, and then used for immunization. Furthermore, by linking antigens to other molecules if necessary, they can be converted into soluble antigens. When transmembrane molecules such as receptors are used as antigens, portions of the extracellular regions of the receptors can be used as fragments, or cells expressing transmembrane molecules on their cell surface may be used as immunogens.

Antibody-producing cells can be obtained by immunizing animals using the suitable 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 as the animals for immunization, where rodents, lagomorphas, and primates are generally used. Examples of such animals include mice, rats, and hamsters for rodents, rabbits for lagomorphas, and monkeys including cynomolgus monkeys, rhesus monkeys, hamadryas, and chimpanzees for primates. In addition, transgenic animals carrying human antibody gene repertoires are also known, and human antibodies can be obtained by using these animals (see WO96/34096; Mendez *et al.*, Nat. Genet. 1997, 15: 146-56). Instead of using such transgenic animals, for example, desired human antibodies having binding activity to desired antigens can be obtained by sensitizing *in vitro* human lymphocytes with the 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. H1-59878 (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 WO93/12227, WO92/03918, WO94/02602, WO96/34096, and WO96/33735).

Animal immunization can be carried out by appropriately diluting and suspending a sensitizing antigen in Phosphate-Buffered Saline (PBS), physiological saline or such, and forming an emulsion by mixing an adjuvant if necessary, followed by an intraperitoneal or subcutaneous injection into animals. After that, 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 target antibody titer 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 using conventional fusing agents (for example, polyethylene glycol) to generate hybridomas (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 antibodies produced from these hybridomas can be measured using known analysis methods such as immunoprecipitation, radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA). Thereafter, hybridomas that produce antibodies in which specificity, affinity, or activity of interest 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). Cloning from mRNAs using RT-PCR is also possible. 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; and IgA-1 and IgA-2). H chains and L chains used in the present invention to produce antibodies are not particularly limited and may derive from antibodies belonging to any of these classes or subclasses; however, IgGs are particularly preferred.

Herein, it is possible to modify H-chain-encoding genes and L-chain-encoding genes using genetic engineering techniques. Genetically modified antibodies such as chimeric antibodies and humanized antibodies that have been artificially modified to decrease heterologous immunogenicity against humans, and such, can be appropriately produced if necessary for antibodies such as mouse antibodies, rat antibodies, rabbit antibodies, hamster antibodies, sheep antibodies, and camel antibodies. Chimeric antibodies are antibodies composed of H chain and L chain variable regions of a nonhuman mammal antibody such as mouse antibody, and the H chain and L chain constant regions of a human antibody. They can be obtained by linking DNAs encoding variable regions of a mouse antibody to DNAs encoding the constant regions of a human antibody, incorporating them into an expression vector, and introducing the vector into a host for production of the antibodies. A humanized antibody, which is also called a reshaped human antibody, can be obtained as follows: A DNA sequence designed to link the complementarity determining regions (CDRs) of an antibody of a nonhuman mammal such as a mouse is synthesized by PCR from a number of oligonucleotides produced so that they have overlapping portions at the ends of the sequence. The obtained DNA can be linked to a DNA encoding a human antibody constant region. The linked DNA can be incorporated into an expression vector, and the vector can be introduced into a host to produce the antibody (see EP239400 and WO96/02576). Human antibody FRs that are linked via the CDRs are selected

so that the CDRs form a favorable antigen-binding site. If necessary, amino acids in the framework regions of antibody variable regions may be substituted such that the CDRs of a reshaped human antibody form an appropriate antigen-binding site (K. Sato *et al.*, Cancer Res. 1993, 53: 851-856).

5 In addition to the humanization described above, antibodies may be modified to improve their biological properties, for example, binding activity to an antigen. Modifications in the present invention can be carried out using methods such as site-directed mutagenesis (see, for example, Kunkel (1985) Proc. Natl. Acad. Sci. USA 82: 488), PCR mutagenesis, and cassette mutagenesis. In general, mutant antibodies whose biological properties have been improved
10 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%, 99% and so on), when compared to the amino acid sequences of the original antibody variable regions. 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
15 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. Generally, naturally-occurring amino acid residues are classified into groups based on the characteristics of their side chains: (1) hydrophobic: alanine, isoleucine, valine, methionine, and leucine; (2) neutral hydrophilic: asparagine, glutamine, cysteine, threonine, and
20 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.

Ordinarily, a total of six complementarity determining regions (CDRs; hypervariable regions) present in the H chain and L chain variable regions interact to form the antigen binding
25 site(s) of an antibody. Even one of these variable regions is known to have the ability to recognize and bind to the antigen, although the affinity will be lower than when all binding sites are included. Therefore, antibody genes of the present invention encoding H chains and L chains only have to encode fragment portions having each of the antigen binding sites of H chains and L chains, and polypeptides encoded by these genes only have to maintain binding
30 activity to the desired antigens.

Heavy chain variable regions are ordinarily composed of three CDR regions and four FR regions as described above. In a preferred embodiment of the present invention, amino acid residues subjected to "modification" can be appropriately selected from among amino acid
residues positioned in the CDR regions or FR regions. Generally, modification of the amino
35 acid residues in the CDR regions can decrease the binding activity to antigens. Therefore, in the present invention, amino acid residues subjected to "modification" are not particularly

limited but are preferred to be appropriately selected from among amino acid residues positioned in the FR regions. Even amino acid residues in the CDRs may be selected, as long as modifications of these residues have been confirmed not to reduce the binding activity.

Furthermore, sequences that can be used as variable region FRs of the antibodies of organisms such as humans or mice, can be appropriately obtained by those skilled in the art using public databases.

The present invention also relates to polypeptides comprising an FcRn-binding domain whose pharmacokinetics in blood is controlled by a method of the present invention.

In a preferred embodiment, the present invention provides humanized antibodies whose pharmacokinetics in blood is controlled by a method of the present invention. For example, the human antibodies are humanized antibodies comprising complementarity determining regions (CDRs) derived from a nonhuman animal, framework regions (FRs) derived from a human, and human constant regions, with at least one amino acid residue in the CDRs or FRs, which can be exposed on antibody surface, has a charge different from that of the corresponding amino acid residue in the wild-type CDRs or FRs, and whose variable regions are derived from an antibody of the nonhuman animal and pharmacokinetics in blood is controlled as compared to corresponding chimeric antibodies having the same constant regions.

The above-described "human constant region" preferably refers to a region comprising a wild-type human Fc domain; however, it may be a modified Fc.

The present invention also relates to methods for producing polypeptides comprising an FcRn-binding domain, whose pharmacokinetics in blood is controlled by using the methods of the present invention. Specifically, the present invention provides methods for producing polypeptides comprising an FcRn-binding domain, whose pharmacokinetics in blood is controlled. The present invention also includes polypeptides comprising an FcRn-binding domain, which are produced by the methods of the present invention.

In a preferred embodiment, the production methods of the present invention are methods for producing polypeptides comprising an FcRn-binding domain, whose pharmacokinetics in blood is controlled, and which comprise:

- (a) modifying the nucleic acids encoding polypeptides comprising an FcRn-binding domain to change the charge of at least one amino acid residue that can be exposed on the surface of the polypeptides;
- (b) culturing host cells to express the nucleic acids; and
- (c) collecting the polypeptides comprising an FcRn-binding domain from the host cell cultures.

The phrase "modifying nucleic acids" in the above-mentioned methods of the present invention refers to modifying nucleic acids so that they correspond to amino acid residues introduced by the "modifications" of the present invention. More specifically, it refers to

modifying the nucleic acids encoding the original (pre-modified) amino acid residues to the nucleic acids encoding the amino acid residues that are to be introduced by the modification. Ordinarily, it means performing a gene manipulation or a mutation treatment that would result in at least one nucleotide insertion, deletion, or substitution to an original nucleic acid so that

5 codons encoding amino acid residues of interest is formed. More specifically, codons encoding the original amino acid residues are substituted with codons encoding the amino acid residues that are to be introduced by the modification. Such nucleic acid modifications can be suitably performed by those skilled in the art using known techniques such as site-directed mutagenesis and PCR mutagenesis.

10 Furthermore, nucleic acids of the present invention are usually carried by (inserted into) suitable vectors and then introduced into host cells. These vectors are not particularly limited so long as the inserted nucleic acids are stably maintained. For example, when using *E. coli* as the host, the cloning vector is preferably pBluescript vector (Stratagene) and such, but various commercially available vectors may be used. Expression vectors are particularly useful as

15 vectors for producing the polypeptides of the present invention. Expression vectors are not particularly limited so long as they can express polypeptides in test tubes, *E. coli*, cultured cells, or individual organisms. For example, preferred vectors include pBEST vector (Promega) for expression in test tubes, pET vector (Invitrogen) for *E. coli*, pME18S-FL3 vector (GenBank Accession No. AB009864) for cultured cells, and pME18S vector (Mol. Cell Biol. 8: 466-472

20 (1998)) for individual organisms. Insertion of a DNA of the present invention into vectors can be performed by standard methods such as ligase reactions 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-mentioned host cells are not particularly limited, and various host cells can

25 be used, depending on the purpose. Cells used for expressing the polypeptides include bacterial cells (for example, *Streptococcus*, *Staphylococcus*, *E. coli*, *Streptomyces*, and *Bacillus subtilis*), fungal cells (for example, yeast and *Aspergillus*), insect cells (for example, *Drosophila* S2 and *Spodoptera* SF9), animal cells (for example, CHO, COS, HeLa, C127, 3T3, BHK, HEK293, and Bowes melanoma cell), and plant cells. Vectors can be introduced into host cells using known

30 methods such as the calcium phosphate precipitation method, electroporation method (Current protocols in Molecular Biology edit. Ausubel *et al.* (1987) Publish. John Wiley & Sons. Section 9.1-9.9), lipofection method, and microinjection method.

For secreting host cell-expressed polypeptides into the lumen of the endoplasmic reticulum, periplasmic space, or extracellular environment, suitable secretion signals can be

35 incorporated into the polypeptides of interest. These signals may be intrinsic or foreign to the polypeptides of interest.

When the polypeptides of the present invention are secreted into the culture media, the polypeptides produced by the above-mentioned methods can be harvested by collecting the media. When the polypeptides of the present invention are produced inside cells, first, the cells are lysed, and then these polypeptides are collected.

5 The polypeptides of the present invention can be collected and purified from recombinant cell cultures by using known methods, including ammonium sulfate or ethanol precipitation, acidic extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography, and lectin chromatography.

10 The present invention relates to compositions (pharmaceutical agents) which comprise polypeptides (for example, IgG antibodies) comprising an FcRn-binding domain, whose pharmacokinetics in blood is controlled by the methods of the present invention, and pharmaceutically acceptable carriers.

15 In the present invention, pharmaceutical compositions usually refer to pharmaceutical 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, such pharmaceutical compositions can be used parenterally, as injections which are sterile solutions or suspensions including the compositions along with water or another pharmaceutically acceptable liquid. For example,
20 such compositions may be formulated as unit doses that meet the requirements for the preparation of pharmaceuticals by appropriately combining the compositions with pharmaceutically acceptable carriers or media, specifically with sterile water, physiological saline, a vegetable oil, emulsifier, suspension, surfactant, stabilizer, flavoring agent, excipient, vehicle, preservative, binder or such. In such preparations, the amount of active ingredient is
25 adjusted such that an appropriate dose that falls within a pre-determined range can be obtained.

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

Aqueous solutions for injection include, for example, physiological saline and isotonic solutions containing dextrose or other adjuvants (for example, D-sorbitol, D-mannose,
30 D-mannitol, and sodium chloride). Appropriate solubilizers, for example, alcohols (ethanol and such), polyalcohols (propylene glycol, polyethylene glycol, and such), nonionic surfactants (polysorbate 80TM, HCO-50, and such), may be used in combination.

Oils include sesame and soybean oils. Benzyl benzoate and/or benzyl alcohol can be used in combination as solubilizers. Buffers (for example, phosphate buffer and sodium acetate
35 buffer), soothing agents (for example, procaine hydrochloride), stabilizers (for example, benzyl

alcohol and phenol), and/or antioxidants can also be combined. Prepared injections are generally filled into appropriate ampules.

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

The administration methods can be appropriately selected considering the patient's age and symptoms. The dose of a pharmaceutical composition comprising an antibody or a polynucleotide encoding an antibody 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 doses in the present invention are not limited to the ranges described above. The doses and administration methods vary depending on a patient's weight, age, symptoms and such. Those skilled in the art can select appropriate doses and administration methods considering the factors described above.

The present invention also provides nucleic acids encoding polypeptides that comprise polypeptides (for example, humanized antibodies) comprising an FcRn-binding domain, whose pharmacokinetics in blood is controlled by the methods of the present invention. Furthermore, vectors carrying these nucleic acids are encompassed by the present invention.

The present invention also provides host cells carrying the above described nucleic acids. 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 or the polypeptides of the present invention. *In vitro* and *in vivo* production systems are available for polypeptide production systems. Production systems that use eukaryotic cells or prokaryotic cells are examples of *in vitro* production systems.

Eukaryotic cells that can be used as a host cell include, for example, animal cells, plant cells, and fungal cells. Animal cells include mammalian cells such as CHO (J. Exp. Med. (1995) 108: 945), COS, HEK293, 3T3, myeloma, BHK (baby hamster kidney), HeLa, and Vero; amphibian cells such as *Xenopus laevis* oocytes (Valle, *et al.* (1981) Nature 291: 338-340); and insect cells such as Sf9, Sf21, and Tn5. In the expression of the antibodies of the present invention, CHO-DG44, CHO-DX11B, COS7 cells, HEK293 cells, and BHK cells can be suitably used. Among animal cells, CHO cells are particularly preferable for large-scale expression. Vectors can be introduced into a host cell by, for example, calcium phosphate methods, DEAE-dextran methods, methods using cationic liposome DOTAP (Boehringer-Mannheim), electroporation methods, or lipofection methods.

Plant cells include, for example, *Nicotiana tabacum*-derived cells and duckweed (*Lemna minor*) known as a protein production system. Calluses can be cultured from these cells to produce the antibodies of the present invention. Known protein production systems are those using fungal cells including 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 prokaryotic production systems. Examples of bacterial cells include *Bacillus subtilis* as well as *E. coli* described above. Such cells can be used to produce the antibodies of the present invention.

When producing an antibody using a host cell of the present invention, the polynucleotide encoding an antibody of the present invention may be expressed by culturing the host cell transformed with an expression vector containing the polynucleotide. The culture can be performed using known methods. For example, when using animal cells as a host, DMEM, MEM, RPMI 1640, or IMDM may be used as the culture medium, and may be used with or without serum supplements such as FBS or fetal calf serum (FCS). The preferred pH is about 6 to 8 during the culture. The culture is carried out typically at a temperature of about 30 to 40°C for about 15 to 200 hours. Medium is exchanged, aerated, or agitated, as necessary.

On the other hand, production systems using animals or plants may be used as systems for producing polypeptides *in vivo*. For example, 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.

Animals to be used for production systems include mammals and insects. Mammals such as goats, pigs, sheep, mice, and cattle may be used (Vicki Glaser SPECTRUM Biotechnology Applications (1993)). Alternatively, the mammals may be transgenic animals.

For example, a polynucleotide encoding an antibody of the present invention may be prepared as a fusion gene with a gene encoding a polypeptide specifically produced in milk, such as the goat β -casein gene. Polynucleotide fragments containing the fusion gene are injected into goat embryos, which are then transplanted into female goats. The desired antibody can be obtained from milk produced by the transgenic goats born from the goats that received the embryos, or from their offspring. Appropriate hormones may be administered to the transgenic goats to increase the volume of milk containing the antibody produced by the goats (Ebert *et al.*, Bio/Technology 12: 699-702 (1994)).

Insects such as silkworms, may also be used for producing the antibodies of the present invention. Baculoviruses carrying a polynucleotide encoding an antibody of interest can be

used to infect silkworms, and the antibody of interest can be obtained from the body fluids of the silkworms (Susumu *et al.*, Nature 315: 592-594 (1985)).

Plants used for producing the antibodies of the present invention include, for example, tobacco. When tobacco is used, a polynucleotide encoding an antibody of interest is inserted
 5 into a plant expression vector, for example, pMON 530, and then the vector is introduced into a bacterium such as *Agrobacterium tumefaciens*. The bacteria are then used to infect tobacco such as *Nicotiana tabacum*, and the desired antibody can be obtained from the leaves (Ma *et al.*, Eur. J. Immunol. 24: 131-138 (1994)). Alternatively, duckweed (*Lemna minor*) is infected with similar bacteria and then cloned. The desired antibody can be obtained from the cloned
 10 duckweed cells (Cox KM *et al.* Nat. Biotechnol. 2006 Dec; 24(12): 1591-1597).

The resulting antibody may be isolated from the inside or outside (such as the medium and milk) of host cells, and purified as a substantially pure and homogenous antibody. Methods for isolating and purifying antibodies are not limited to any specific method and any standard method for isolating and purifying polypeptides may be used. Antibodies can be isolated and
 15 purified, by appropriately selecting or combining, for example, chromatographic columns, filtration, ultrafiltration, salting out, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectric focusing, dialysis, recrystallization, and others.

Chromatographies include, for example, affinity chromatographies, ion exchange
 20 chromatographies, hydrophobic chromatographies, gel filtrations, reverse-phase chromatographies, and adsorption chromatographies (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak *et al.*, Cold Spring Harbor Laboratory Press, 1996). These chromatographies can be carried out using liquid phase chromatographies such as HPLC and FPLC. Examples of affinity chromatography columns
 25 include protein A columns and protein G columns. Examples of the proteins A columns include Hyper D, POROS, and Sepharose F. F. (Pharmacia).

An antibody can be modified freely and peptide portions can be deleted from it by treating the antibody with an appropriate protein modifying enzyme before or after antibody purification, as necessary. Such protein modifying enzymes include, for example, trypsins,
 30 chymotrypsins, lysyl endopeptidases, protein kinases, and glucosidases.

The above-described methods for producing polypeptides of the present invention comprising an FcRn-binding domain, whose pharmacokinetics in blood is controlled, which comprise culturing host cells of the present invention and collecting the polypeptides from the cell cultures, are also preferred embodiments of the present invention.

35 All prior art documents cited herein are incorporated herein by reference.

Examples

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

5 [Example 1] Humanization of bispecific antibody

A bispecific antibody consisting of the combination of the anti-Factor IXa antibody A69-VH, anti-Factor X antibody B26-VH, and hybrid L chain (BBA), which was found to be most effective in shortening blood coagulation time in Japanese Patent Application No. 2005-112514, was humanized as described below.

10

1-1. Homology search of humanized antibodies

Using a database constructed by obtaining amino acid sequence data of human antibodies from the publicly-disclosed Kabat Database (<ftp://ftp.ebi.ac.uk/pub/databases/kabat/>) and IMGT Database (<http://imgt.cines.fr/>), homology search was carried out separately for the mouse A69-H chain variable region (amino acid sequence; SEQ ID NO: 15), mouse B26-H chain variable region (amino acid sequence; SEQ ID NO: 16), and mouse BBA-L chain variable region (amino acid sequence; SEQ ID NO: 17). The results confirmed that they have high homologies to the human antibody sequences shown below, and thus it was decided that they would be used as the framework regions (hereinafter abbreviated as FRs) of humanized antibodies.

20

(1) A69-H chain variable region: KABATID-000064 (Kabat Database)

(Kipps *et al.*, J. Clin. Invest. 1991; 87:2087-2096)

(2) B26-H chain variable region: EMBL Accession No. AB063872 (IMGT Database)
(Unpublished data)

25 (3) BBA-L chain variable region: KABATID-024300 (Kabat Database)

(Welschhof *et al.*, J. Immunol. Method 1995; 179:203-214)

Humanized antibodies in which complementarity determining regions (hereinafter abbreviated as CDRs) of each mouse antibody were grafted into the FRs of the human antibodies (1)-(3) were prepared.

30

Also, the web homology search site publicly disclosed by NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to search secretory signal sequences of human antibodies that are highly homologous to the human antibodies of (4)-(6). The following secretory signal sequences obtained by the search were used.

(4) A69-H chain variable region: GenBank Accession No. AF062257

35 (5) B26-H chain variable region: GenBank Accession No. AAC18248

(6) BBA-L chain variable region: GenBank Accession No. AAA59100

1-2. Construction of humanized antibody gene expression vector

Twelve synthetic oligoDNAs of about 50 bases were prepared from a nucleotide sequence encoding the amino acid sequence from a secretory signal sequence to an antibody variable region, such that about 20 bases of their 3'-end anneal with each other. The synthetic oligoDNAs were designed so that the 5'-terminal nucleotides encode a human sequence, the 3'-terminal nucleotides encode a mouse sequence, or all nucleotides encode human sequences. Furthermore, a primer annealing to the 5'-end of an antibody variable region gene and having the XhoI cleavage sequence, and a primer annealing to the 3'-end of an antibody variable region gene, having the SfiI cleavage sequence and also encoding the 5'-end sequence of an intron sequence were prepared.

1 μ l each of the synthetic oligoDNAs prepared at 2.5 μ M were mixed, and 1x TaKaRa Ex Taq Buffer, 0.4 mM dNTPs, and 0.5 units TaKaRa Ex Taq (all from Takara) were added to prepare 48 μ l of a reaction solution. After heating this at 94°C for 5 minutes, 2 cycles of reacting at 94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 2 minutes were performed to assemble and elongate each of the synthetic oligoDNAs. Next, 1 μ l (10 μ M each) of primers annealing to the 5'-end and to the 3'-end of the antibody gene were added, and the antibody variable region genes were amplified by 35 cycles of reacting at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 min and then reacting at 72°C for 5 minutes. After PCR, the whole amount of the reaction solution was subjected to 1% agarose gel electrophoresis. Amplified fragments having the size of interest (approximately 400 bp) were purified using the QIAquick Gel Extraction Kit (QIAGEN) according to the method described in the instruction manual, and were eluted with 30 μ l of sterile water. These fragments were cloned using the pGEM-T Easy Vector System (Promega) according to the method described in the instruction manual. The nucleotide sequence of each of the DNA fragments was determined using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3730xL DNA Sequencer or ABI PRISM 3700 DNA Sequencer (Applied Biosystems) according to the method described in the instruction manual.

The H-chain variable region fragment-inserted plasmid and the L-chain variable region fragment-inserted plasmid, each of which was confirmed to have a correct humanized antibody variable region gene sequence, were digested with XhoI and SfiI, and EcoRI respectively. Next, the reaction solution was subjected to 1% agarose gel electrophoresis. DNA fragments having the size of interest (approximately 400 bp) were purified using QIAquick Gel Extraction Kit (QIAGEN) according to the method described in the instruction manual, and eluted with 30 μ l of sterile water. Then, expression vectors for animal cells were prepared as follows. To preferentially express IgG4 whose H chains are of a heterologous combination, a CH3 portion

amino acid-substituted IgG4 was used by referring to the knobs-into-holes technique of IgG1 (Merchant AM *et al.*, Nature Biotechnology, Vol.16, p.677-681 (1998)). Furthermore, to promote H chain dimer formation, amino acid substitution (-ppcpScp- → -ppcpPcp-) was also introduced to the hinge. Humanized A69 H chain expression vector was prepared by inserting
 5 a humanized A69 H chain variable region antibody gene fragment into an expression vector prepared by inserting Y349C and T366W-substituted constant region gene to pCAGGS comprising a chicken β -actin promoter (Niwa *et al.*, Gene Vol.108, p.193-199 (1991)). In addition, humanized B26 H chain expression vector was prepared by inserting a humanized B26 H chain variable region antibody gene fragment into an expression vector prepared by inserting
 10 E356C, T366S, L368A, and Y407V-substituted constant region gene to pCAGGS. The plasmid (pCAG-gkDNA) prepared by inserting a wild type antibody L chain constant region into pCAGGS was digested with EcoRI to prepare an expression vector inserted with a humanized BBA L chain variable region antibody gene fragment. Ligation reaction was performed using Rapid DNA Ligation Kit (Roche Diagnostics), and DH5 α strain *E. coli* (TOYOBO) was
 15 transformed.

1-3. Expression of the humanized bispecific antibody

The humanized bispecific antibody was expressed according to the following method. Human fetal renal carcinoma cell-derived HEK293H strain (Invitrogen) was suspended in
 20 DMEM medium (Invitrogen) containing 10% Fetal Bovine Serum (Invitrogen), and 10 ml of this was seeded at a cell density of $5-6 \times 10^5$ cells/ml in each dish used for adhesive cells (10-cm diameter, CORNING) and cultured for one day in a CO₂ incubator (37°C, 5% CO₂). Then, the medium was removed by suction, and 6.9 ml of CHO-S-SFM-II medium (Invitrogen) containing 1% Fetal Bovine Serum (Invitrogen) was added. The plasmid DNA mixture solution prepared
 25 in Example 1-2 (total of 13.8 μ g) was mixed with 20.7 μ l of 1 μ g/ml Polyethylenimine (Polysciences Inc.) and 690 μ l of CHO-S-SFMII medium, left to stand at room temperature for 10 minutes, then the cells were added to the cells in each dish and incubated in a CO₂ incubator (37°C, 5% CO₂) for 4-5 hours. Thereafter, 6.9 ml of CHO-S-SFM-II medium (Invitrogen) containing 1% Fetal Bovine Serum (Invitrogen) was added and then the cells were incubated in a
 30 CO₂ incubator for 3 days. The culture supernatant was recovered, then cells were removed by centrifugation (at approximately 2000 g for 5 minutes at room temperature), and the solution was sterilized by passing it through a 0.22 μ m filter MILLEX®-GV (Millipore). The sample was stored at 4°C until use.

35 1-4. Purification of the humanized bispecific antibody

100 µl of rProtein A SepharoseTM Fast Flow (Amersham Biosciences) was added to the culture supernatant obtained according to the method described in Example 1-2, and the solution was mixed by inversion at 4°C for 4 hours or more. The solution was transferred to a 0.22 µm filter cup, Ultrafree[®]-MC (Millipore), and after washing 3 times with 500 µl of TBS containing 0.01% Tween[®] 20, the rProtein A SepharoseTM resin was suspended in 100 µl of 50 mM sodium acetate solution containing 0.01% Tween[®] 20 at pH 3.3 and left to stand for 2 minutes. Then, the antibody was eluted, and the eluate was immediately neutralized by adding 6.7 µl of 1.5 M Tris-HCl, pH 7.8.

10 1-5. Quantification of humanized bispecific antibody concentration

The antibody concentration was determined using the following two types of methods.

Goat anti-human IgG (Biosource International) was adjusted to 1 µg/ml with a coating buffer, and immobilized to a Nunc-Immuno plate (Nunc). After blocking with a diluent buffer (D.B.), a sample of the culture supernatant suitably diluted with D.B. was added. Furthermore, as a standard for calculating the antibody concentration, human IgG4 (humanized anti-TF antibody, see WO 99/51743) diluted with D.B. in a three-fold dilution series up to eleven stages starting from 2000 ng/ml was added similarly. After three washes, a goat anti-human IgG, alkaline phosphatase (Biosource International) was reacted. After five washes, the color was developed using Sigma 104[®] phosphatase substrate (Sigma-Aldrich) as a substrate, and the absorbance at 405 nm was measured on an absorbance reader Model 3550 (Bio-Rad Laboratories) with a reference wavelength of 655 nm. Using the Microplate Manager III (Bio-Rad Laboratories) software, human IgG concentration in the culture supernatant was calculated from the standard curve.

Furthermore, the antibody concentration was quantified using Sensor Chip CM5 (BIACORE) to which Protein A had been immobilized, with Biacore 1000 or Biacore Q (BIACORE). More specifically, Protein A-immobilized sensor chip was prepared according to the manufacturer's protocol by reacting an activated sensor chip with a Protein A solution (SIGMA) diluted to 50 µg/ml with 10 mM aqueous sodium acetate solution (pH 4.0, BIACORE) at 5 µl/min for 30 minutes, and then performing a blocking operation. This sensor chip was used to measure the concentration of the culture supernatant and the purified product using Biacore 1000 (BIACORE). HBS-EP Buffer (BIACORE) was used for the immobilization of the sensor chip and for the measurements of concentration. As a standard for concentration measurements, humanized IgG4 antibody (humanized anti-tissue factor antibody, see WO 99/51743) diluted with HBS-EP Buffer in a two-fold dilution series up to six stages beginning at 4000 ng/ml was used.

1-6. Assessment of blood coagulation activity of humanized bispecific antibody

To elucidate whether a bispecific antibody corrects the coagulation ability of hemophilia A blood, effects of the bispecific antibody on activated partial thromboplastin time (APTT) were examined using Factor VIII-deficient plasma. A mixed solution comprising 50 μ l of an antibody solution at various concentrations, 50 μ l of Factor VIII-deficient plasma (Biomerieux), and 50 μ l of APTT reagent (Dade Behring) was heated at 37°C for 3 minutes. Coagulation reaction was initiated by adding 50 μ l of 20 mM CaCl_2 (Dade Behring) to this mixed solution. The time required for coagulation was measured with CR-A (Amelung)-connected KC10A (Amelung).

Using a calibration curve provided by defining the coagulation time of Factor VIII-deficient plasma as 0% and that of normal plasma as 100%, Factor VIII-like activity (%) of a bispecific antibody was calculated from the coagulation time measured when the bispecific antibody was added.

1-7. Preparation of humanized bispecific antibody retaining blood coagulation activity

For humanized bispecific antibodies which had reduced blood coagulation ability in the above-described blood coagulation activity assessment, amino acids of their human antibody FRs were modified to increase their activities. Specifically, mutations were introduced to the humanized antibody variable region using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the method described in the instruction manual. The H-chain variable region fragment-inserted plasmid and L-chain variable region fragment-inserted plasmid were confirmed to have the humanized antibody variable region gene sequences of interest and were digested with XhoI and SfiI, and EcoRI respectively. The reaction solution was subjected to 1% agarose gel electrophoresis. DNA fragments having the size of interest (approximately 400 bp) were purified using a QIAquick Gel Extraction Kit (QIAGEN) according to the method described in the instruction manual, and eluted with 30 μ l of sterile water. Then, expression vectors for animal cells were prepared according to the method described in Example 1-2. A humanized bispecific antibody was prepared according to the method described in Examples 1-3, 1-4, and 1-5 and blood coagulation activity was evaluated according to the method described in Example 1-6.

By repeated amino acid modifications of the FR sequences and assessment of blood coagulation ability, a humanized bispecific antibody (humanized A69 (hA69a)/humanized B26 (hB26-F123e4)/humanized BBA (hAL-F123j4)) having the same level of activity as the chimeric bispecific antibody (A69/B26/BBA) was obtained (Fig. 1). Each of the antibody variable region sequences is indicated in the following SEQ ID NOs.

(1) humanized A69 antibody VH (hA69a) SEQ ID NO: 1 (nucleotide sequence), SEQ ID NO: 2 (amino acid sequence)

(2) humanized B26 antibody VH (hB26-F123e4) SEQ ID NO: 3 (nucleotide sequence), SEQ ID NO: 4 (amino acid sequence)

5 (3) humanized BBA antibody VL (hAL-F123j4) SEQ ID NO: 5 (nucleotide sequence), SEQ ID NO: 6 (amino acid sequence)

[Example 2] Selection of amino acid modification sites in variable regions to isolate a bispecific antibody

10 Antibody Fv region models for humanized A69 and B26 antibodies were prepared by homology modeling using MOE software (Chemical Computing Group Inc.) to confirm amino acid residues exposed on the surface of variable regions of these antibodies. The models are shown in Fig. 2. A detailed analysis of the models suggested that among surface-exposed amino acids in the FR sequences other than CDRs, those at H10, H12, H23, H39, H43, and H105
15 (according to the Kabat's numbering; Kabat EA *et al.* 1991. Sequences of Proteins of Immunological Interest. NIH) were candidate amino acids that could be modified to shift the isoelectric point without reduction of activity. The amino acid at H97 was selected as a surface-exposed amino acid in CDRs.

20 [Example 3] Modification of variable region amino acid sequences of humanized A69 antibody and modified form thereof, and humanized B26 antibody

Amino acids of the H chain variable regions of humanized A69 and B26 antibodies were modified to shift the isoelectric points of the antibodies. Specifically, mutations were introduced into the H chain variable regions of humanized A69 antibody (hA69a; nucleotide
25 SEQ ID NO: 1) and humanized B26 antibody (hB26-F123e4; nucleotide SEQ ID NO: 3) prepared using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the method described in the attached instruction manual. H chain variable region fragment-inserted plasmids which had been confirmed to have the sequence of humanized antibody variable region gene of interest were digested with XhoI and SfiI, and then the reaction mixtures were subjected
30 to electrophoresis using 1% agarose gel. DNA fragments having the size of interest (about 400 bp) were purified using a QIAquick Gel Extraction Kit (QIAGEN) according to the method described in the attached instruction manual, and then eluted using 30 µl of sterile water. The DNA fragments were inserted into an expression plasmid carrying the wild type constant region by the method described in Example 1-2 to construct H chain expression vectors. Modified
35 amino acid residues in the respective antibodies and their SEQ IDs are shown in Table 1. (hA69-N97R, hA69-p18), and humanized B26 antibody (hB26-F123e4) and its modified form

(hB26-p15) were prepared. A humanized A69 antibody (hA69a) and its modified forms (hA69-N97R, hA69-p18) were expressed using a combination of H chain expression vectors (variable region is hA69-N97R, hA69-p18) and an L chain expression vector (variable region is hAL-F123j4; SEQ ID NO: 6) according to Example 1-3. Meanwhile, a humanized B26 antibody (hB26-F123e4) and its modified form (hB26-p15) were expressed using a combination of H chain expression vectors (variable region is hB26-F123e4, hB26-p15) and an L chain expression vector (variable region is B26-VL; the amino acid sequence is described in WO2005/035756 (SEQ ID NO: 18)) according to Example 1-3. The antibodies were purified from culture supernatants by the method described in Example 1-4.

Table 1

Name	Humanized A69-H chain variable region	
	Modification site	Amino acid SEQ ID NO:
hA69a	-	2
hA69-p18	Q43E, Q105E	7
hA69-N97R	N97R	9

Name	Humanized B26-H chain variable region	
	Modification site	Amino acid SEQ ID NO:
hB26-F123e4	-	4
hB26-p15	Q39K, Q43K, Q105R	10

- 15 [Example 4] Establishment of a cell line that expresses a bispecific antibody derived from humanized A69 or B26 antibodies

To prepare a humanized bispecific antibody, an antibody-expressing cell line was established by the procedure described below.

- 20 An H-chain constant region was amplified by PCR using a wild-type human IgG4 H-chain constant region gene as a template and using a 5'-end primer designed so that the nucleotide sequence encoding two amino acids (Ala-Ser) in the N-terminal side of the H-chain constant region will be an NheI recognition sequence (GCTAGC) and a primer that anneals to the 3'-end and that carries a NotI recognizing site. Then, the amplified fragments were ligated to pBluescriptKS+ vector (TOYOBO) digested with NheI and Not I (both from Takara) to prepare pBCH4 (comprising an IgG4 constant region gene). PCR was performed using primers which are complementary to the 5'-end nucleotide sequence of the H-chain variable regions of

the humanized A69-H chain antibody (hA69-PFL: SEQ ID NO: 11) and humanized B26-H chain antibody (hB26-PF: SEQ ID NO: 12) and which have a Kozak sequence (CCACC) and an EcoRI recognition sequence, and a primer on the 3'-end nucleotide sequence having an NheI recognition sequence. The obtained PCR products were digested with EcoRI and NheI (both from Takara) and inserted into pBCH4 also digested with EcoRI and NheI, and then the variable regions and the constant regions were linked. The prepared vector for humanized A69-H chain antibody was digested with EcoRI and NotI (both Takara), and then cloned into the animal cell expression vector pCXND3 digested with EcoRI and NotI. The procedure for the construction of the vector pCXND3 is described below.

DHFR-ΔE-rVH-PM1-f (see WO92/19759) was cleaved at the EcoRI and SmaI restriction sites to separate the vector backbone from the antibody H chain gene. Only the vector backbone was recovered, and then an EcoRI-NotI-BamHI adaptor (Takara) was cloned into it. The resulting vector was named pCHOI. The DHFR gene expression region derived from pCHOI was cloned into pCXN (Niwa *et al.*, Gene 108: 193-200 (1991)) at the HindIII restriction site. The resulting vector was named pCXND3. In addition, the prepared vector for humanized B26-H chain antibody was digested with EcoRI and NotI (both Takara), and then cloned into the animal cell expression vector pCXZD1 digested with EcoRI and NotI. pCXZD1 vector is an expression vector obtained from pCXND3 by substituting the Zeocin resistance gene for the neomycin resistance gene. Furthermore, an L-chain expression vector was prepared by inserting the L chain variable region of the humanized BBA-L chain antibody (hAL-s8; SEQ ID NO: 8) into a plasmid (pCAG-gkDNA) having an inserted L chain constant region according to Example 1-2. The prepared three types of expression vectors were linearized with restriction enzymes and then introduced into CHO-DG44 cells to establish an antibody-expressing cell line.

A stable expression cell line was prepared by the procedure described below. Genes were introduced by electroporation using GenePulserXcell (Bio-Rad). Each antibody expression vector was combined with 0.75 ml of CHO cells suspended in PBS (1×10^7 cells/ml). The mixtures were cooled on ice for ten minutes, transferred into cuvettes, and pulsed at 1.5 kV and 25 μ FD. After a ten-minute restoration period at room temperature, the electroporated cells were suspended in 40 ml of CHO-S-SFMII medium (Invitrogen) containing 1x HT supplement (Invitrogen). The suspension was diluted 10 times with the same medium and aliquoted into 96-well culture plates at 100 μ l/well. After 24 hours of culture in a CO₂ incubator (5% CO₂), Geneticin and Zeocin (both Invitrogen) were added at 0.5 mg/ml and 0.6 mg/ml, respectively. The cells were cultured for two weeks. Expansion cultures were sequentially performed for colonies of drug-resistant transformants. An established high expression cell line was used for large scale culture to obtain the culture supernatant.

[Example 5] Separation and purification of humanized antibody homodimers and a humanized bispecific antibody

Using the method described below, the bispecific antibody was purified from the culture supernatants obtained in Example 4. The culture supernatants were loaded onto an rProtein A Sepharose Fast Flow column (Amersham Biosciences; 50 mm I.D. x 9.9 cm H. = 194.3 ml resin) equilibrated with an equilibration buffer (20 mmol/l sodium phosphate buffer, 150 mol/l NaCl, pH 7.0). After washing with wash buffer 1 (20 mmol/l sodium phosphate buffer, 150 mol/l NaCl, pH 7.0) and wash buffer 2 (50 mmol/l sodium acetate buffer, pH 6.0), the column was eluted with 50 mmol/l acetic acid. Immediately after elution, pH was adjusted to 6.3 by adding 1.5 mol/l Tris-HCl (pH 7.8).

The resulting purified solution was loaded onto an SP TOYOPEARL 650M column (Tosoh; 26 mm I.D. x 22.3 cm H. = 118.3 ml resin) equilibrated with Solvent A (10 mmol/l sodium phosphate buffer, pH 6.3). The antibodies were separated based on their surface charges using the solutions and gradients indicated below.

Solvent A: 20 mmol/l sodium acetate buffer (pH 6.0)

Solvent B: 20 mmol/l sodium acetate buffer, 1 mol/l NaCl (pH 6.0)

Flow rate: 10 ml/min (113 cm/h); 5.3 ml/min (60 cm/h) only at the time of elution

20	Gradient: 0 → 15% B	stepwise	3 column volumes (CV)
	15 → 35% B	gradient	6 CV
	35 → 50% B	gradient	10 CV
	50 → 100% B	gradient	3 CV
	100% B	stepwise	4 CV

Two types of homodimers (hA69-PF and hB26-PF) and a single type of heterodimer, the bispecific antibody BiAb, were obtained by collecting eluted fractions of the detected three peaks separately.

[Example 6] Analysis of prepared antibodies by isoelectric focusing

ATF is a previously obtained monoclonal antibody against human tissue factor, and is a humanized antibody comprising the constant region of human IgG4. The origin of ATF is described in detail in WO99/051743. The amino acid sequences of its H chain and L chain variable regions are shown in SEQ ID Nos: 13 and 14, respectively. hA69-PF, BiAb, and hB26-PF prepared in Example 5; hA69-N97R, hA69-p18, hB26-e, and hB26-p15 prepared in Example 3; and ATF were analyzed by isoelectric focusing to assess changes in the surface charge due to the following: differences in the amino acid sequences of their variable regions and amino acid modifications.

ATF, hA69-PF, BiAb, hB26-PF, and the humanized A69 antibody hA69-N97R and a modified form thereof, hA69-p18, as well as the humanized B26 antibody hB26-F123e4 and a modified form thereof, hB26-p15, were subjected to isoelectric focusing, as described below. Using Phastsystem Cassette (Amersham Bioscience), Phast-Gel Dry IEF gel (Amersham Bioscience) was allowed to swell for about 30 minutes in the swelling solution indicated below.

Milli-Q water 1.5 ml

Pharmalyte 5-8 for IEF (Amersham Bioscience) 50 μ l

Pharmalyte 8-10.5 for IEF (Amersham Bioscience) 50 μ l

Electrophoresis was carried out in PhastSystem (Amersham Bioscience) using the swollen gel according to the program indicated below. The samples were loaded onto the gel in Step 2. The pI marker used is a calibration kit for pI (Amersham Bioscience).

Step 1: 2000 V 2.5 mA 3.5 W 15°C 75 Vh

Step 2: 200 V 2.5 mA 3.5 W 15°C 15 Vh

Step 3: 2000 V 2.5 mA 3.5 W 15°C 410 Vh

After electrophoresis, the gel was fixed with 20% TCA, and then silver-stained using a silver staining kit protein (Amersham Bioscience) according to the protocol attached to the kit. After staining, the pIs of the samples were calculated from the known pIs of the pI marker. The analysis result of isoelectric focusing is shown in Fig. 3. The calibration curve of pI vs mobility prepared using the pI marker and pIs calculated from the curve are shown in Fig. 4. The pIs were calculated based on the mobility of major bands since each sample exhibited antibody-derived charge heterogeneity.

The result showed that the surface charges were changed due to differences in the amino acid sequences of the variable regions and that the pIs were shifted due to the change in surface charge through amino acid modifications. The pIs were as follows: about 9.2 for hB26-PF, about 8.7 for BiAb, about 8.0 for hA69-PF, about 7.2 for ATF, about 8.9 for hA69-N97R, about 8.5 for hA69-p18, about 8.7 for hB26-F123e4, and about 9.0 for hB26-p15. hA69-N97R, hA69-p18, and hA69-PF were obtained by modifying the same humanized antibody variable region. A pI shift of about 0.9 could be achieved in hA69-PF compared with hA69-N97R, and a pI shift of about 0.3 could be achieved in hB26-p15 compared with hB26-F123e4. The examination described above demonstrates that pI can be shifted depending on the amino acid sequence of a variable region as well as by modifying a surface amino acid at H10, H12, H23, H39, H43, H97, or H105 in a selected variable region to change its charge.

[Example 7] Assessment of humanized antibodies A69 and B26 and modified forms thereof for their binding activity

The functions of the humanized A69 antibody and its modified form were assessed by assaying their binding activities to the antigen Factor IXa, as described below. Humanized A69 antibody (hA69a) and its modified form, hA69-N97R, were assessed by the following procedure. Factor IXa β (Enzyme Research Laboratories) diluted to 1 μ g/ml with a coating buffer (100 mM sodium bicarbonate (pH 9.6), 0.02% sodium azide) was aliquoted (100 μ l/well) into a Nunc-Immuno plate (a Nunc-ImmunoTM 96 MicroWellTM MaxiSorpTM plate (Nalge Nunc International)), and then the plate was incubated overnight at 4°C. After washing three times with PBS(-) containing Tween^(R) 20, the plate was blocked with a diluent buffer (50 mM Tris-HCl (pH 8.1), 1% bovine serum albumin, 1 mM MgCl₂, 0.15 M NaCl, 0.05% Tween^(R) 20, 0.02% sodium azide) at room temperature for two hours. After removal of the buffer, the purified antibodies diluted with the diluent buffer were added to the plate at 100 μ l/well. Then, the plate was incubated at room temperature for one hour. After the plate was washed three times, alkaline phosphatase-labeled goat anti-mouse IgG (BIOSOURCE) diluted to 1/4000 with the diluent buffer was added at 100 μ l/well. Then, the plate was incubated at room temperature for one hour. After washing the plate five times, a chromogenic substrate (Sigma) was added at 100 μ l/well. The plate was then incubated at room temperature for 30 minutes. Absorbance at 405 nm (reference at 655 nm) was measured using the Model 3550 Microplate Reader (Bio-Rad Laboratories).

The modified antibodies (hA69-N97R, hA69-p18, and hA69-PF) used in Example 8 were assessed by the following procedure. After Factor IXa (Enzyme Research Laboratories) diluted to 1 μ g/ml with a coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) was aliquoted (100 μ l/well) into a Nunc-Immuno plate (a Nunc-ImmunoTM 96 MicroWellTM MaxiSorpTM plate (Nalge Nunc International)), the plate was incubated at 4°C overnight or for a longer period. After washing three times with PBS containing 0.05% Tween^(R) 20, a diluent buffer (Tris buffered saline containing Tween 20 (pH 8.0) (Sigma), 1% bovine serum albumin, 0.02% sodium azide) was added to the plate at 200 μ l/well. Then, the plate was blocked at room temperature for two hours. After removal of the buffer, the purified antibodies diluted with the diluent buffer were added at 100 μ l/well. The plate was then incubated overnight at 4°C. After washing the plate three times, alkaline phosphatase-labeled mouse anti-human IgG4 (Southern Biotechnology)) diluted to 1/500 with the diluent buffer was added at 100 μ l/well. The plate was incubated at room temperature for two hours. After washing the plate five times, the BluePhos Microwell Phosphatase Substrates System (Kirkegaard & Perry Laboratories) was added as a substrate at 100 μ l/well. The plate was then incubated at room temperature for about 30 minutes. Absorbance at 650 nm was measured using the Vmax Microplate Reader (Molecular Devices). As shown in Fig. 5, the results demonstrate that the antibodies in which

the variable region had been modified to change the surface charge showed a binding activity comparable to that of the original antibodies before modification.

Furthermore, the functions of the humanized B26 antibody hB26-F123e4 and its modified form, hB26-p15, were assessed by assaying their binding activities to the antigen Factor X. Factor X (Enzyme Research Laboratories) diluted to 1 µg/ml with a coating buffer (100 mM sodium bicarbonate (pH 9.6), 0.02% sodium azide) was aliquoted (100 µl/well) into a Nunc-Immuno plate (a Nunc-Immuno™ 96 MicroWell™ MaxiSorp™ plate), and then the plate was incubated overnight at 4°C. After washing three times with PBS(-) containing Tween^(R) 20, the plate was blocked with a diluent buffer (50 mM Tris-HCl (pH 8.1), 1% bovine serum albumin, 1 mM MgCl₂, 0.15 M NaCl, 0.05% Tween^(R) 20, 0.02% sodium azide) at room temperature for two hours. After removal of the buffer, the purified antibodies diluted with the diluent buffer were added at 100 µl/well to the plate. The plate was incubated at room temperature for one hour. After the plate was washed three times, alkaline phosphatase-labeled goat anti-mouse IgG (BIOSOURCE) diluted to 1/4000 with the diluent buffer was added at 100 µl/well. The plate was then incubated at room temperature for one hour. After washing the plate five times, a chromogenic substrate (Sigma) was added at 100 µl/well. The plate was then incubated at room temperature for 30 minutes. Absorbance at 405 nm (reference at 655 nm) was measured using the Model 3550 Microplate Reader (Bio-Rad Laboratories). As shown in Fig. 6, the results demonstrated that the antibody in which the variable region had been modified to change the surface charge showed binding activity comparable to that of the original antibody before modification.

The findings described above demonstrate that the modifications of variable regions performed in the Examples have no influence on the antigen binding activity of the antibodies.

25 [Example 8] Assessment of the prepared antibodies for pharmacokinetics

8-1. Test of pharmacokinetics using mice

ATF was obtained as a monoclonal antibody against human tissue factor, and is a humanized antibody comprising the constant regions of human IgG4. The origin of ATF is described in detail in WO99/051743. The amino acid sequences of its H chain and L chain variable regions are shown in SEQ ID NOs: 13 and 14, respectively. hA69-PF, BiAb, and hB26-PF prepared in Example 5, hA69-N97R, hA69-p18, hB26-e, and hB26-p15 prepared in Example 3, and ATF were assessed for the *in vivo* kinetics in mice (C57BL/6J; Charles River Japan, Inc.). ATF, hA69-PF, BiAb, and hB26-PF were intravenously administered once at 5 mg/kg to mice (C57BL/6J; Charles River Japan, Inc.). The blood was collected before administration and 15 minutes, two hours, eight hours, and one, two, four, seven, 11, 14, 21, and 28 days after administration. The collected blood was immediately centrifuged at 4°C and

15,000 rpm for 15 minutes to obtain plasma. The separated plasma was stored in a freezer at -20°C or below until use. Likewise, hA69-N97R, hA69-p18, hB26-F123e4, and hB26-p15 were intravenously administered once at 1 mg/kg to mice (C57BL/6J; Charles River Japan, Inc.). The blood was collected before administration and 15 minutes, two hours, eight hours, and one, two, five, seven, nine, 14, 21, and 28 days after administration. The collected blood was immediately centrifuged at 4°C and 15,000 rpm for 15 minutes to obtain plasma. The separated plasma was stored in a freezer at -20°C or below until use.

8-2. Measurement of plasma concentration by ELISA

Plasma concentrations in mice were determined by ELISA. Calibration curve samples of 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, and 0.1 µg/ml plasma concentrations were prepared. The standard curve samples and mouse plasma samples to be tested were aliquoted into immunoplates (Nunc-Immuno MaxiSorp plates (Nalge nunc International) immobilized with an anti-human IgG (γ-chain specific) F(ab')₂ (Sigma). The samples were left to stand at room temperature for one hour, and then reacted with Goat Anti-Human IgG-BIOT (Southern Biotechnology Associates) and Streptavidin-alkaline phosphatase conjugate (Roche Diagnostics) in succession. The color development was carried out using BluePhos Microwell Phosphatase Substrates System (Kirkegaard & Perry Laboratories) as a substrate. The absorbance at 650 nm was measured using a microplate reader. The plasma concentrations in mice were calculated from the absorbance on the calibration curve using the analysis software SOFTmax PRO (Molecular Devices). The time courses of plasma concentrations of ATF, hA69-PF, BiAb, and hB26-PF are shown in Fig. 7.

8-3. Method for calculating pharmacokinetic data

The obtained data on the time courses of plasma concentrations were evaluated by a model-independent analysis using the pharmacokinetic analysis software WinNonlin (Pharsight) to calculate pharmacokinetic parameters (clearance (CL), half-life (T_{1/2})). T_{1/2} was calculated from plasma concentrations at the last three points or in the terminal phase automatically selected by WinNonlin. The determined pharmacokinetic parameters are shown in Table 2.

Table 2

	hA69-N97R	hA69-p18	hA69-PF	ATF
pI	8.9	8.5	8.0	7.2
CL mL/h/kg	0.412	0.300	0.204	0.136
T1/2 day	12.6	15.0	18.7	26.1

	hB26-F123e4	hB26-p15	hB26-PF	BiAb
pI	8.7	9.0	9.2	8.7
CL mL/h/kg	0.346	0.450	0.600	0.362
T1/2 day	13.4	11.9	10.8	13.6

- 5 Furthermore, plots of antibody clearance (CL) and half-life (T1/2) relative to pI are shown in Fig. 8. While the respective antibodies used share the same constant region sequences, each of clearance (CL) and half-life (T1/2) is strongly correlated with pI. This shows that, as pI is lower, clearance is lower and half-life in blood is more prolonged. Thus, half-life in blood can be controlled by pI values even when antibodies share the same constant region sequences.
- 10 Accordingly, it is suggested that the half-life in blood can be prolonged by decreasing pI or can be reduced by increasing pI. In this Example, it is demonstrated that the half-life in blood could be actually prolonged by decreasing pI through modification of surface amino acids (the modification sites are shown in Table 3) in the variable regions of hA69-N97R. Half-life in blood can be reduced by increasing pI through modification of surface amino acids (the
- 15 modification sites are shown in Table 4) in the variable regions of hB26-F123e4. These findings suggest that pharmacokinetics of IgGs in blood can be controlled by changing charges of surface amino acids (for example, at positions H10, H12, H23, H39, H43, H97, and H105) in their variable regions through modifications.

20

Table 3

Name	H1	H12	H23	H27	H43	H97	H103	L99
hA69-N97R	Pyr(Q)	K	K	G	Q	R	Q	G
hA69-p18	Pyr(Q)	K	K	G	E	N	E	G
hA69-PF	E	V	T	Y	E	L	E	Q
		*	*		*	*	*	

Table 4

Name	H1	H9	H10	H28	H37	H39	H43	H103	L99
hB26-F123e4	Pyr(Q)	P	D	M	A	Q	Q	Q	G
hB26-p15	Pyr(Q)	P	D	M	A	K	K	R	G
hB26-PF	E	A	Q	T	V	Q	K	R	Q
			*			*	*	*	

In Tables 3 and 4 above, Pyr(Q) represents an N-terminal glutamine residue which is assumed to be pyroglutamylated. Since the N-terminal amino group is protected, there is no significant charge difference between Pyr(Q) and E. Furthermore, sites of amino acid substitution which results in a pI shift are indicated by an asterisk.

The present invention discovered that the half-life in blood of an IgG could be prolonged or reduced by decreasing or increasing the pI of IgG through substitution of surface amino acids in the variable regions, respectively.

According to a non-patent document (Nat Biotechnol. 1997; 15: 637-640) on blood pharmacokinetics in mice, the half-life in blood (T_{1/2}) could be prolonged by about 1.5 times by increasing the affinity for FcRn through modification of amino acids in the Fc in the constant region. Also in the present invention, by decreasing pI through modification of surface amino acids in variable regions, the half-life in blood (T_{1/2}) could be prolonged by about 1.5 times in the comparison between hA69-N97R and hA69-PF sharing the same constant region sequences. Furthermore, when hA69-N97R is compared with hA69-PF and ATF, T_{1/2} of ATF with the lowest pI is longer by about 2.1 times than that of hA69-N97R. Thus, the half-life of hA69-N97R in blood can be further prolonged by decreasing its pI through additional modification of surface amino acids in the variable regions of hA69-N97R. When the antibodies used in this Example are compared to each other, the half-life in blood is different by about 2.4 times between hB26-PF with the highest pI and ATF with the lowest pI. Accordingly, the control of pharmacokinetics in blood through amino acid modifications in variable regions is expected to be more effective as compared to previous control techniques. Furthermore, the number of amino acid substitutions artificially introduced into constant regions is desired to be smaller from the viewpoint of immunogenicity. Thus, the present invention, in which half-life in blood is controlled by modifying surface amino acids in variable regions, is expected to be useful in developing pharmaceuticals.

Industrial Applicability

In a preferred embodiment of the methods of the present invention, amino acid substitutions are performed in variable regions, and thus the risk of immunogenicity is low as

compared to conventional methods that modify constant regions. Furthermore, the methods of the present invention can be more effective in prolonging the half-life in blood as compared to the conventional methods that modify constant regions. In addition, the half-life in blood of polypeptides comprising an FcRn-binding domain, such as IgG antibodies, can be controlled by
5 controlling the surface charge in variable regions without changing structure or function (activity). Polypeptides comprising an FcRn-binding domain, which retain the original activity and whose half-life in blood is controlled, can be obtained by using the methods of the present invention.

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CLAIMS:

1. A method for producing a polypeptide comprising an FcRn-binding domain, whose pharmacokinetics in blood is controlled, wherein the method comprises:

(a) modifying a nucleic acid encoding a polypeptide comprising an FcRn-binding domain to change the charge of at least one amino acid residue that can be exposed on the surface of the polypeptide;

(b) culturing a host cell to express the nucleic acid;

(c) collecting the polypeptide comprising an FcRn-binding domain from the host cell culture; and

(d) comparing the blood pharmacokinetics of the polypeptide comprising an FcRn-binding domain to that of the polypeptide before the modification of the amino acid residue,

wherein the amino acid residue that can be exposed on the surface of the polypeptide comprising an FcRn-binding domain is located in a domain other than the FcRn-binding domain within the polypeptide.

2. The method of claim 1, wherein the method further comprises:

(e) culturing a host cell comprising a nucleic acid encoding the polypeptide comprising an FcRn-binding domain; and

(f) collecting the polypeptide comprising an FcRn-binding from the host cell culture.

3. The method of claim 2, wherein the FcRn-binding domain comprises an Fc or Fc-like domain.

25 4. The method of claim 1, wherein the polypeptide comprising an FcRn-binding domain is an IgG antibody.

30 5. The method of claim 4, wherein the amino acid residue whose charge is changed in step (a) is an amino acid residue in a heavy chain or light chain variable region of the IgG antibody.

6. The method of claim 1, wherein the control of pharmacokinetics in blood is the control of any one of the following parameters: half-life in blood, mean residence time in blood, or blood clearance.

7. The method of claim 1, wherein the change of charge of the amino acid residue in step (a) is achieved by an amino acid substitution.
8. A polypeptide comprising an FcRn-binding domain, which is produced by the method of claim 1.
9. A method for controlling blood pharmacokinetics of a polypeptide comprising an FcRn-binding domain, which comprises changing the charge of at least one amino acid residue that can be exposed on the surface of the polypeptide and comparing the blood pharmacokinetics of the polypeptide comprising an FcRn-binding domain to that of the polypeptide before the modification of the amino acid residue,
wherein the amino acid residue that can be exposed on the surface of the polypeptide comprising an FcRn-binding domain is located in a domain other than the FcRn-binding domain within the polypeptide.
10. The method of claim 9, wherein the FcRn-binding domain comprises an Fc or Fc-like domain.
11. The method of claim 9, wherein the polypeptide comprising an FcRn-binding domain is an IgG antibody.
12. The method of claim 11, wherein the amino acid residue whose charge is changed is an amino acid residue in a heavy chain or light chain variable region of the IgG antibody.
13. The method of claim 9, wherein the control of pharmacokinetics in blood is the control of any one of the following parameters: half-life in blood, mean residence time in blood, or blood clearance.
14. The method of claim 9, wherein the change of charge of the amino acid residue is achieved by an amino acid substitution.

15. A method for producing a humanized antibody comprising complementarity determining regions (CDRs) of a nonhuman animal, human-derived framework regions (FRs), and human constant regions, whose pharmacokinetics in blood is controlled, wherein the method comprises:

(a) modifying, at the time of humanization or after humanization, at least one amino acid residue that can be exposed on the surface in the CDRs or FRs so as to have a charge different from that of the corresponding amino acid residue in CDRs or FRs of the nonhuman antibody, and

(b) comparing the blood pharmacokinetics of the humanized antibody to that of the chimeric antibody whose variable regions are derived from an antibody of the nonhuman animal and whose constant regions are the same.

16. A method of claim 15, wherein the method further comprises:

(c) culturing a host cell comprising a nucleic acid encoding the humanized antibody;
and

(d) collecting the humanized antibody from the host cell culture.

17. A method for producing an IgG antibody, whose pharmacokinetics in blood is controlled, wherein the method comprises:

(a) changing the charge of at least one amino acid residue selected from the amino acid residues at positions 10, 12, 23, 39, 43, and 105 according to the Kabat's numbering system in a heavy chain variable region, and

(b) comparing the blood pharmacokinetics of the IgG antibody to that of the IgG antibody before the modification of the amino acid residue.

18. A method of claim 17, wherein the method further comprises:

(c) culturing a host cell comprising a nucleic acid encoding the IgG antibody; and

(d) collecting the IgG antibody from the host cell culture.

Chugai Seiyaku Kabushiki Kaisha

Patent Attorneys for the Applicant/Nominated Person

SPRUSON & FERGUSON

FIG. 1

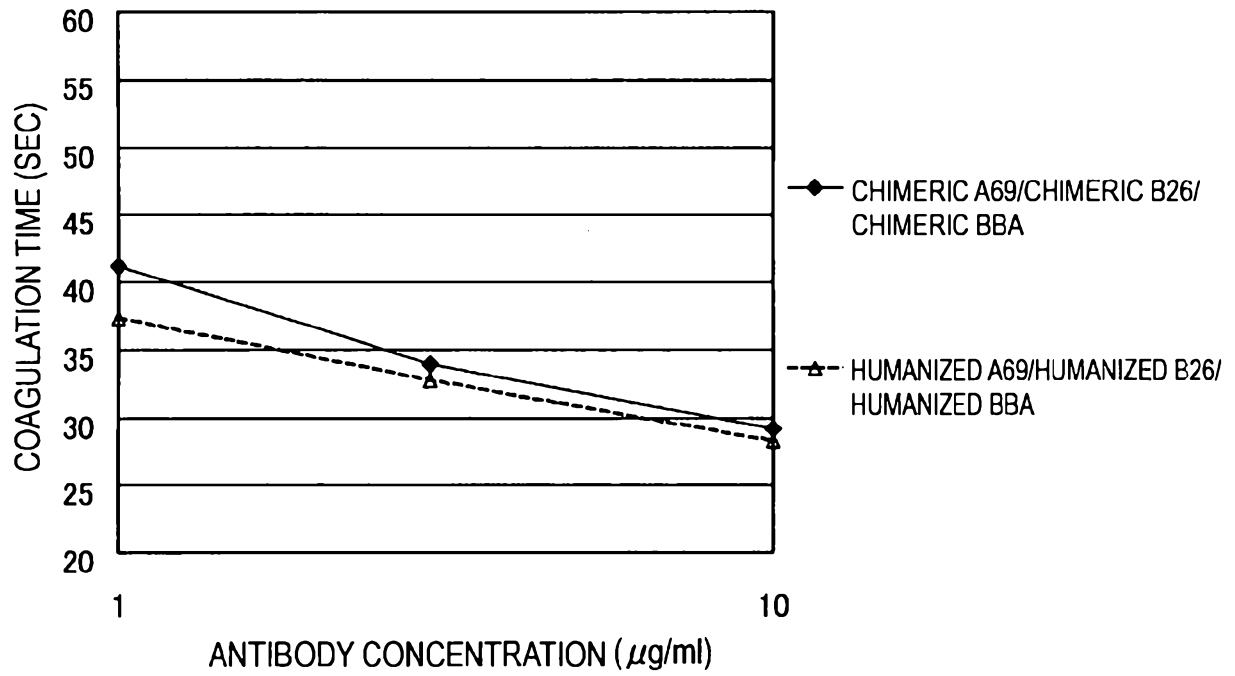
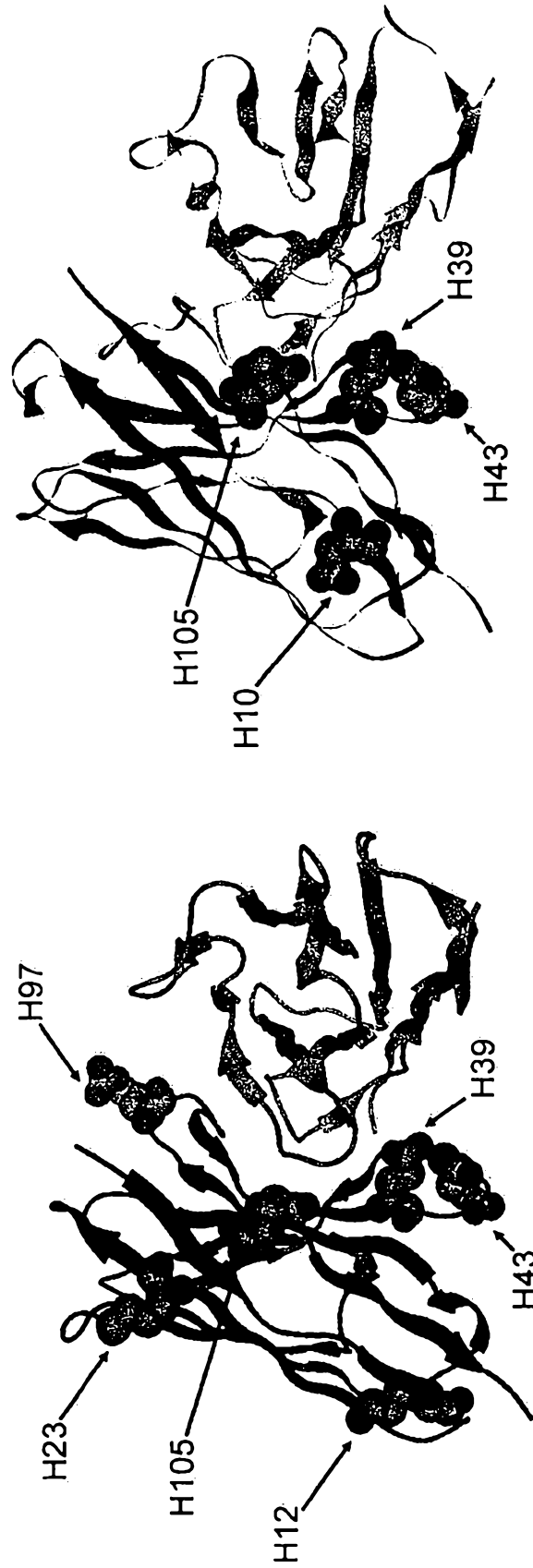


FIG. 2



HUMANIZED B26 ANTIBODY

HUMANIZED A69 ANTIBODY

FIG. 3

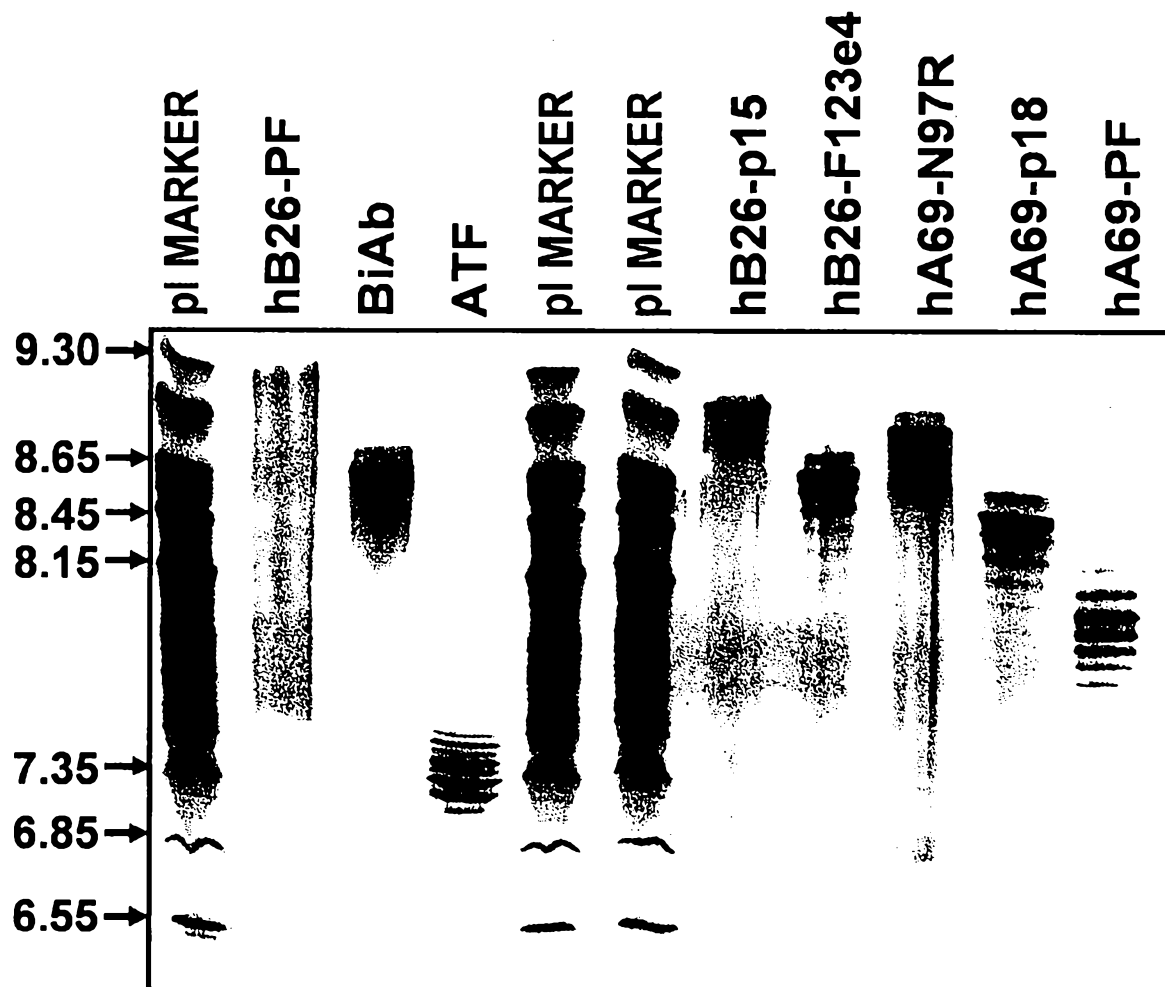
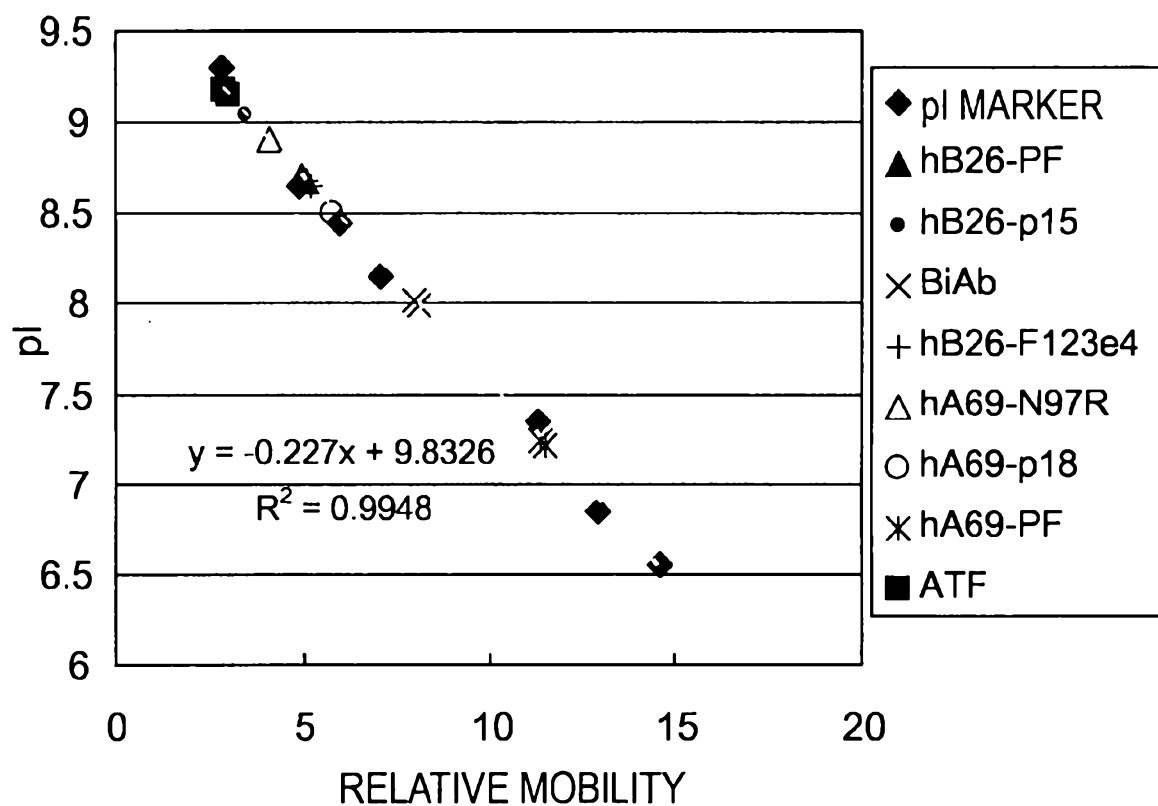


FIG. 4



SAMPLE	pI
hB26-PF	9.2
hB26-p15	9.0
BiAb	8.7
hB26-F123e4	8.7
hA69-N97R	8.9
hA69-p18	8.5
hA69-PF	8.0
ATF	7.2

FIG. 5

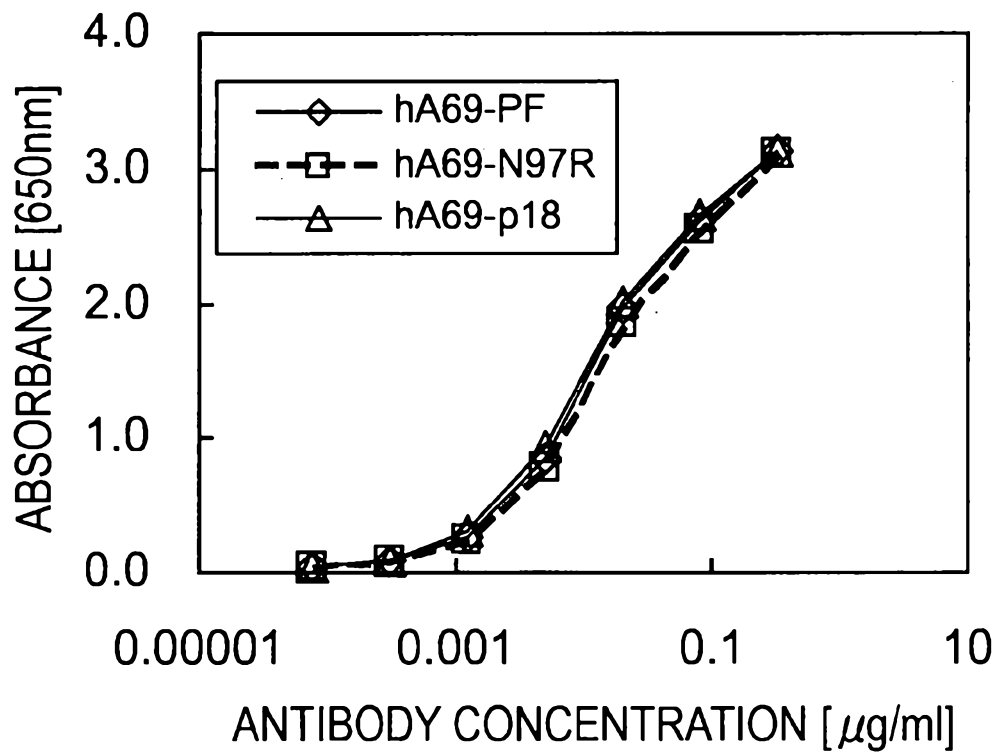
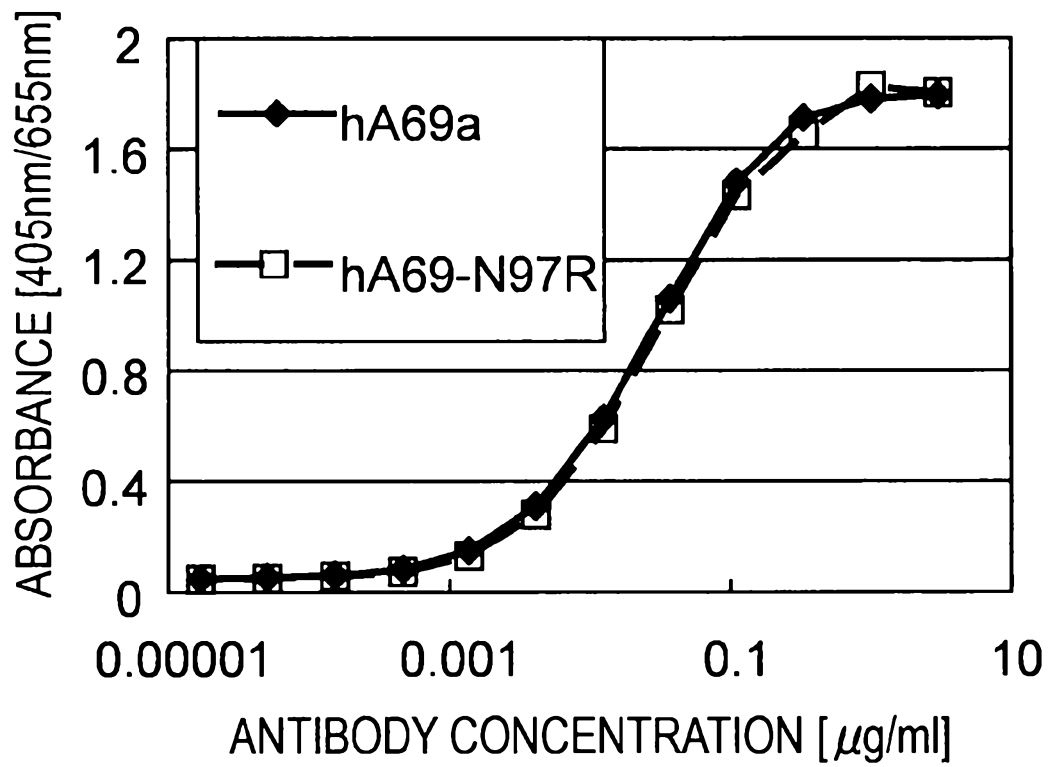


FIG. 6

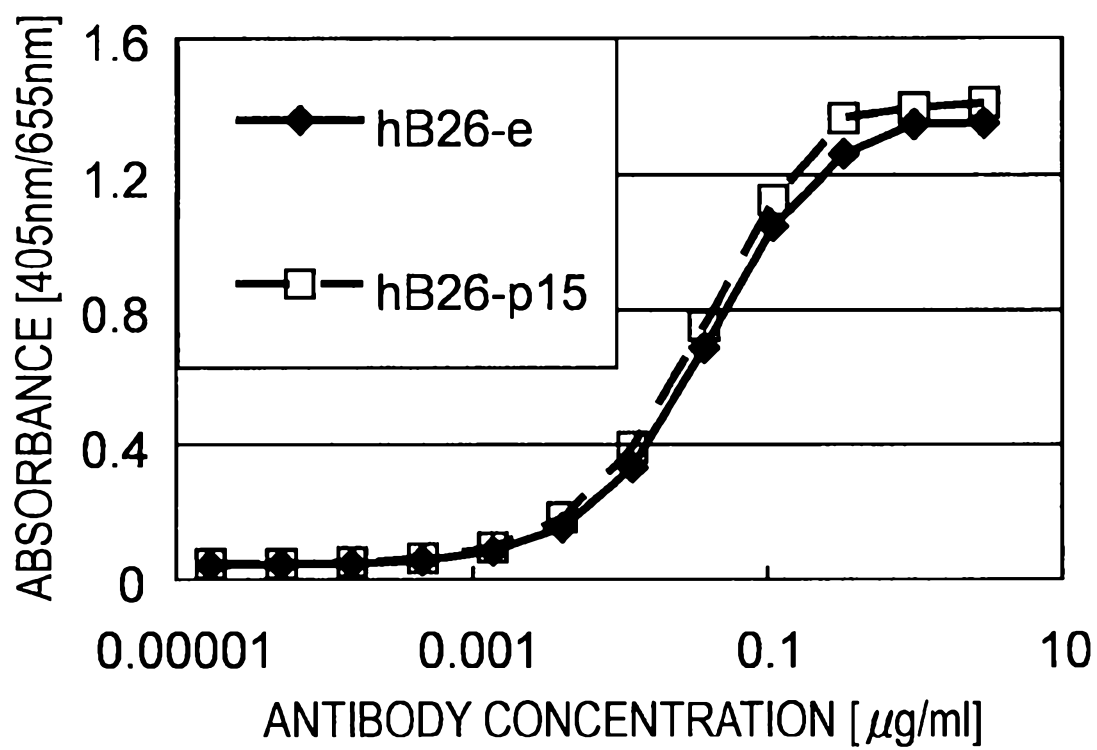


FIG. 7

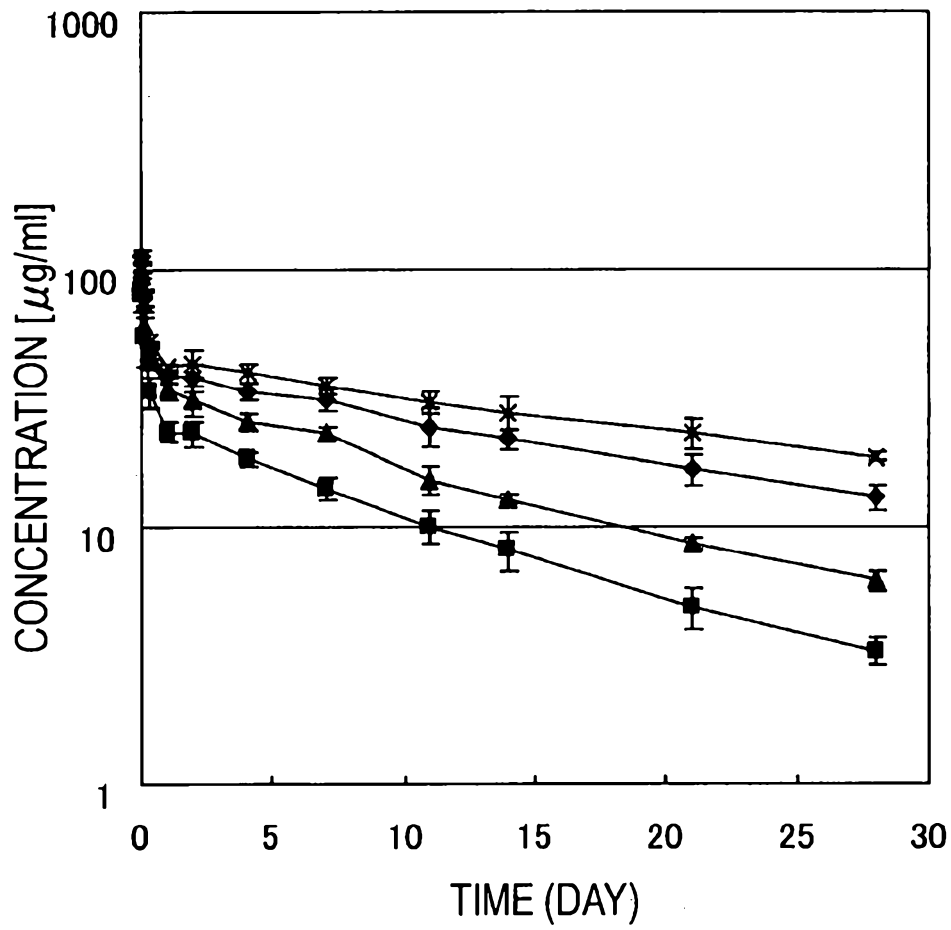
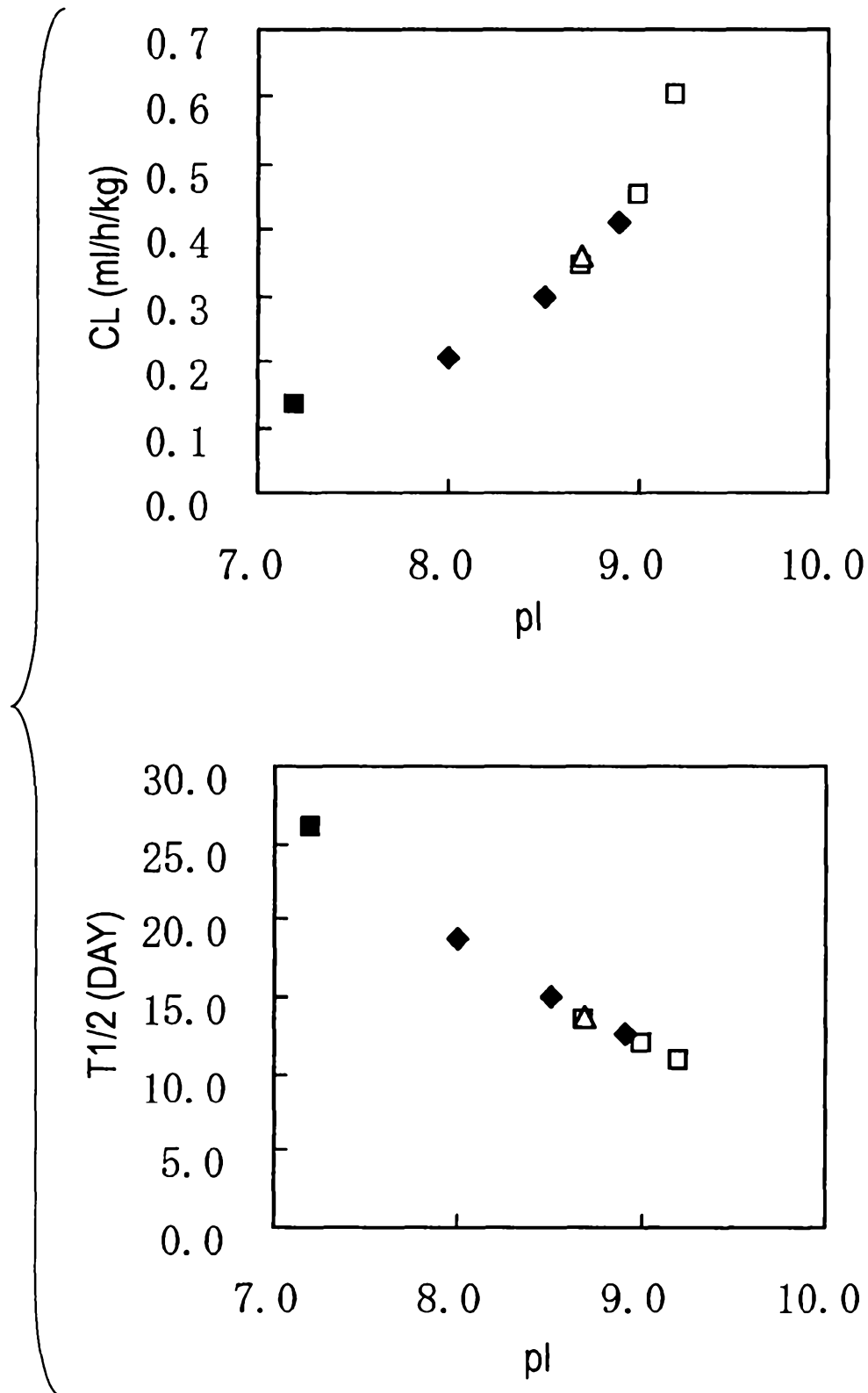


FIG. 8



SEQUENCE LISTING

<110> CHUGAI SEIYAKU KABUSHIKI KAISHA

<120> Methods for regulating antibody kinetics in blood

<130> C1-A0605P

<150> JP 2006-097796

<151> 2006-03-31

<160> 18

<170> PatentIn version 3.3

<210> 1

<211> 354

<212> DNA

<213> Artificial

<220>

<223> An artificially synthesized nucleotide sequence

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tcctgcaagg cctctggagg caccttcagt gactactata tgcactgggt gcgccaggcc 120

cccggacaag ggcttgagtg gatgggatac attaatccta gcagtgggta tactaagtac 180

aatcggaagt tcagggacag agtcaccatt accgcggaca aatccacgag cacagcctac 240

atggagctga gcagcctgag atctgaagac acggctgtgt attactgtgc gagaggggggt 300

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<210> 2

<211> 118

<212> PRT

<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 2

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

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

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

Gly Tyr Ile Asn Pro Ser Ser Gly Tyr Thr Lys Tyr Asn Arg Lys Phe
 50 55 60
 Arg Asp Arg Val Thr Ile Thr Ala Asp Lys Ser Thr Ser Thr Ala Tyr
 65 70 75 80
 Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Gly Gly Asn Gly Tyr Tyr Leu Asp Tyr Trp Gly Gln Gly Thr
 100 105 110
 Thr Val Thr Val Ser Ser
 115

<210> 3
 <211> 357
 <212> DNA
 <213> Artificial

<220>
 <223> An artificially synthesized nucleotide sequence

<400> 3
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 tcctgcaagg cctctggcta catgttttcc gacaacaaca tggactgggc gcgacaggcc 120
 cctggacaag ggcttgagtg gatgggagat attaatacta aaagtgggtg ttctatctac 180
 aaccagaagt tcaagggcag agtcatcatg accatagaca aatccacggg cacagcctac 240
 atggaattga ggagcctgag atcagacgac acggccatat attactgtgc gaggaggagg 300
 agctacggct actactttga ctactggggc caggggaaccc tggtcaccgt ctctca 357

<210> 4
 <211> 119
 <212> PRT
 <213> Artificial

<220>
 <223> An artificially synthesized peptide sequence

<400> 4

Gln Val Gln Leu Val Gln Ser Gly Pro Asp Val Lys Lys Pro Gly Ala
 1 5 10 15
 Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Met Phe Ser Asp Asn
 20 25 30
 Asn Met Asp Trp Ala Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
 35 40 45

Gly Asp Ile Asn Thr Lys Ser Gly Gly Ser Ile Tyr Asn Gln Lys Phe
 50 55 60
 Lys Gly Arg Val Ile Met Thr Ile Asp Lys Ser Thr Gly Thr Ala Tyr
 65 70 75 80
 Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Ile Tyr Tyr Cys
 85 90 95
 Ala Arg Arg Arg Ser Tyr Gly Tyr Tyr Phe Asp Tyr Trp Gly Gln Gly
 100 105 110
 Thr Leu Val Thr Val Ser Ser
 115

<210> 5
 <211> 318
 <212> DNA
 <213> Artificial

<220>
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 atcacttgca aggccagtc gaatgtggg actgctgtag cctggatatca gcagaaacca 120
 gggaaagccc ctaagctcct gatctattcg gcacccctacc ggtacagtgg ggtcccatca 180
 aggttcagtg gcagtcgata tgggacagat ttactctca ccatctcaag cttgcaacct 240
 gaagatttag caacttacta ctgtcagcaa tatagcaact atatcacgtt cggcggaggg 300
 accaaggtgg agatcaaa 318

<210> 6
 <211> 106
 <212> PRT
 <213> Artificial

<220>
 <223> An artificially synthesized peptide sequence

<400> 6

Asp Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15
 Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Ala
 20 25 30
 Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
 35 40 45

Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly
 50 55 60
 Ser Arg Tyr Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
 65 70 75 80
 Glu Asp Leu Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Asn Tyr Ile Thr
 85 90 95
 Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
 100 105

<210> 7
 <211> 118
 <212> PRT
 <213> Artificial

<220>
 <223> An artificially synthesized peptide sequence

<400> 7

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser
 1 5 10 15
 Ser Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Asp Tyr
 20 25 30
 Tyr Met His Trp Val Arg Gln Ala Pro Gly Glu Gly Leu Glu Trp Met
 35 40 45
 Gly Tyr Ile Asn Pro Ser Ser Gly Tyr Thr Lys Tyr Asn Arg Lys Phe
 50 55 60
 Arg Asp Arg Val Thr Ile Thr Ala Asp Lys Ser Thr Ser Thr Ala Tyr
 65 70 75 80
 Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Gly Gly Asn Gly Tyr Tyr Leu Asp Tyr Trp Gly Glu Gly Thr
 100 105 110
 Thr Val Thr Val Ser Ser
 115

<210> 8
 <211> 106
 <212> PRT
 <213> Artificial

<220>
 <223> An artificially synthesized peptide sequence

<400> 8

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Asp Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1           5           10           15
Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Ala
20           25           30
Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35           40           45
Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly
50           55           60
Ser Arg Tyr Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65           70           75           80
Glu Asp Leu Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Asn Tyr Ile Thr
85           90           95
Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100          105

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<210> 9

<211> 118

<212> PRT

<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 9

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Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser
1           5           10           15
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Asp Tyr
20           25           30
Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35           40           45
Gly Tyr Ile Asn Pro Ser Ser Gly Tyr Thr Lys Tyr Asn Arg Lys Phe
50           55           60
Arg Asp Arg Val Thr Ile Thr Ala Asp Lys Ser Thr Ser Thr Ala Tyr
65           70           75           80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85           90           95
Ala Arg Gly Gly Arg Gly Tyr Tyr Leu Asp Tyr Trp Gly Gln Gly Thr
100          105          110

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Thr Val Thr Val Ser Ser
115

<210> 10

<211> 119

<212> PRT

<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 10

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

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

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

Gly Asp Ile Asn Thr Lys Ser Gly Gly Ser Ile Tyr Asn Gln Lys Phe
50 55 60

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

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

Ala Arg Arg Arg Ser Tyr Gly Tyr Tyr Phe Asp Tyr Trp Gly Arg Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
115

<210> 11

<211> 118

<212> PRT

<213> Artificial

<220>

<223> An artificially synthesized peptide sequence

<400> 11

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

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

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

Gly Tyr Ile Asn Pro Ser Ser Gly Tyr Thr Lys Tyr Asn Arg Lys Phe
 50 55 60
 Arg Asp Arg Val Thr Ile Thr Ala Asp Lys Ser Thr Ser Thr Ala Tyr
 65 70 75 80
 Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Gly Gly Leu Gly Tyr Tyr Leu Asp Tyr Trp Gly Glu Gly Thr
 100 105 110
 Thr Val Thr Val Ser Ser
 115

<210> 12
 <211> 119
 <212> PRT
 <213> Artificial

<220>
 <223> An artificially synthesized peptide sequence

<400> 12

Glu Val Gln Leu Val Gln Ser Gly Ala Gln Val Lys Lys Pro Gly Ala
 1 5 10 15
 Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Ser Asp Asn
 20 25 30
 Asn Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Met
 35 40 45
 Gly Asp Ile Asn Thr Lys Ser Gly Gly Ser Ile Tyr Asn Gln Lys Phe
 50 55 60
 Lys Gly Arg Val Ile Met Thr Ile Asp Lys Ser Thr Gly Thr Ala Tyr
 65 70 75 80
 Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Ile Tyr Tyr Cys
 85 90 95
 Ala Arg Arg Arg Ser Tyr Gly Tyr Tyr Phe Asp Tyr Trp Gly Arg Gly
 100 105 110
 Thr Leu Val Thr Val Ser Ser
 115

<210> 13
 <211> 463
 <212> PRT
 <213> Homo sapiens

<400> 13

Met	Asp	Trp	Thr	Trp	Arg	Phe	Leu	Phe	Val	Val	Ala	Ala	Ala	Thr	Gly
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Val	Gln	Ser	Gln	Val	Gln	Leu	Leu	Glu	Ser	Gly	Ala	Val	Leu	Ala	Arg
			20					25					30		
Pro	Gly	Thr	Ser	Val	Lys	Ile	Ser	Cys	Lys	Ala	Ser	Gly	Phe	Asn	Ile
		35					40					45			
Lys	Asp	Tyr	Tyr	Met	His	Trp	Val	Lys	Gln	Arg	Pro	Gly	Gln	Gly	Leu
	50					55					60				
Glu	Trp	Ile	Gly	Gly	Asn	Asp	Pro	Ala	Asn	Gly	His	Ser	Met	Tyr	Asp
65					70					75					80
Pro	Lys	Phe	Gln	Gly	Arg	Val	Thr	Ile	Thr	Ala	Asp	Thr	Ser	Thr	Ser
				85					90						95
Thr	Val	Phe	Met	Glu	Leu	Ser	Ser	Leu	Arg	Ser	Glu	Asp	Thr	Ala	Val
			100					105						110	
Tyr	Tyr	Cys	Ala	Arg	Asp	Ser	Gly	Tyr	Ala	Met	Asp	Tyr	Trp	Gly	Gln
		115					120					125			
Gly	Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val
	130					135					140				
Phe	Pro	Leu	Ala	Pro	Cys	Ser	Arg	Ser	Thr	Ser	Glu	Ser	Thr	Ala	Ala
145					150					155					160
Leu	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser
			165						170					175	
Trp	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val
		180						185					190		
Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro
		195					200					205			
Ser	Ser	Ser	Leu	Gly	Thr	Lys	Thr	Tyr	Thr	Cys	Asn	Val	Asp	His	Lys
	210					215					220				
Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Arg	Val	Glu	Ser	Lys	Tyr	Gly	Pro
225					230					235					240
Pro	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Phe	Leu	Gly	Gly	Pro	Ser	Val
				245					250					255	
Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr
			260					265					270		
Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	Gln	Glu	Asp	Pro	Glu
		275					280					285			

Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
 290 295 300
 Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val Val Ser
 305 310 315 320
 Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
 325 330 335
 Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile
 340 345 350
 Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
 355 360 365
 Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
 370 375 380
 Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
 385 390 395 400
 Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
 405 410 415
 Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys Ser Arg
 420 425 430
 Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
 435 440 445
 His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly Lys
 450 455 460

<210> 14
 <211> 236
 <212> PRT
 <213> Homo sapiens

<400> 14

Met Asp Met Arg Val Pro Ala Gln Leu Leu Gly Leu Leu Leu Trp
 1 5 10 15
 Leu Arg Gly Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser
 20 25 30
 Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser
 35 40 45
 Gln Asp Ile Lys Ser Phe Leu Ser Trp Tyr Gln Gln Lys Pro Glu Lys
 50 55 60
 Ala Pro Lys Ser Leu Ile Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val
 65 70 75 80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr
85 90 95

Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln
100 105 110

His Gly Glu Ser Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile
115 120 125

Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp
130 135 140

Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn
145 150 155 160

Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu
165 170 175

Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp
180 185 190

Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr
195 200 205

Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser
210 215 220

Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
225 230 235

<210> 15
<211> 119
<212> PRT
<213> Mus musculus

<400> 15

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

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

Tyr Tyr Met His Trp Ile Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp
35 40 45

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

Phe Arg Asp Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala
65 70 75 80

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

Cys Ala Arg Gly Gly Asn Gly Tyr Tyr Leu Asp Tyr Trp Gly Gln Gly
 100 105 110

Thr Thr Leu Thr Val Ser Ser
 115

<210> 16
 <211> 120
 <212> PRT
 <213> Mus musculus

<400> 16

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

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

Asn Asn Met Asp Trp Val Lys Gln Ser His Gly Lys Gly Leu Glu Trp
 35 40 45

Ile Gly Asp Ile Asn Thr Lys Ser Gly Gly Ser Ile Tyr Asn Gln Lys
 50 55 60

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

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

Cys Ala Arg Arg Arg Ser Tyr Gly Tyr Tyr Phe Asp Tyr Trp Gly Gln
 100 105 110

Gly Thr Thr Leu Thr Val Ser Ser
 115 120

<210> 17
 <211> 107
 <212> PRT
 <213> Homo sapiens

<400> 17

Asp Ile Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val Gly
 1 5 10 15

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

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

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

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

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

Phe Gly Gly Gly Thr Lys Leu Glu Leu Lys Arg
100 105

<210> 18

<211> 108

<212> PRT

<213> Mus musculus

<400> 18

Asp Ile Val Leu Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
1 5 10 15

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

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

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

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

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

Thr Phe Gly Ala Gly Thr Lys Leu Glu Ile Lys Arg
100 105