Description

Title of Invention: PHARMACEUTICAL FORMULATION CONTAINING IMPROVED ANTIBODY MOLECULES

Technical Field

[0001] Present invention relates to a polypeptide and/or antibody-containing formulation, particularly, to a stable liquid formulation containing a high concentration of a modified anti-IL6 receptor antibody.

Background Art

[0002] In recent years, various antibody formulations have been developed and used in practice. Many of those formulations are used for intravenous injection. Designing antibody-containing formulations for subcutaneous injections makes it necessary to increase the antibody concentration in the liquid, since the antibody amount per administration is large (about 100 mg to 200 mg) and the volume for subcutaneous injections is generally limited.

[0003] In solutions containing a high concentration of an antibody, unwanted degradation occurs, which includes the formation of insoluble and/or soluble aggregates. Those insoluble and soluble aggregates are likely to be formed in the liquid state by association of the antibody molecule. In cases when a liquid formulation is stored for a long time, the bioactivity of the antibody molecules might get lost or reduced due to deamidation of aspargine residues. The cycle of freezing and thawing leads to the formation of degraded and aggregated antibody molecules too.

[0004] Various ideas have been proposed for providing a stabilized formulation in which loss of the active component is reduced even after the formulation has been stored for a longer period of time. Such formulations are obtained by dissolving an active component and various additives in a buffer solution. There is a need to provide a high concentrated antibody-containing formulation in which dimerization and deamidation during long-term storage are inhibited, and which is both stable and suitable for use in subcutaneous administration.

[0005] Antibodies are drawing attention as pharmaceuticals as they are highly stable in plasma and have few adverse effects. Among them, a number of IgG-type antibody pharmaceuticals are available on the market and many antibody pharmaceuticals are currently under development (Non Patent Literature 1 and 2). IL-6 is a cytokine involved in various autoimmune diseases, inflammatory diseases, malignant tumors, and so on (Non Patent Literature 3). TOCILIZUMAB, a humanized anti-IL-6 receptor IgG1 antibody, specifically binds to the IL-6 receptor. It is thought that TOCILIZUMAB can be used as a therapeutic agent for IL-6-associated diseases such

as rheumatoid arthritis, since it neutralizes the biological activity of IL-6 (Patent Literature 1 to 3, and Non Patent Literature 4). TOCILIZUMAB has been approved as a therapeutic agent for Castleman's disease and rheumatoid arthritis in Japan (Non Patent Literature 5).

[0006] Humanized antibodies such as TOCILIZUMAB are first-generation antibody pharmaceuticals. Second-generation antibody pharmaceuticals are currently being developed by improving the efficacy, convenience, and cost of first-generation antibody pharmaceuticals. Various technologies that are applicable to second-generation antibody pharmaceuticals are being developed. Technologies for enhancing effector function, antigen-binding ability, pharmacokinetics, and stability, as well as technologies for reducing the risk of immunogenicity have been reported. As methods for enhancing drug efficacy or reducing dosage, technologies that enhance antibody-dependent cell-mediated cytotoxic activity (ADCC activity) or complement-dependent cytotoxic activity (CDC activity) through amino acid substitution in the Fc region of an IgG antibody have been reported (Non Patent Literature 6). Furthermore, affinity maturation has been reported as a technology for enhancing antigen-binding ability or antigen-neutralizing ability (Non Patent Literature 7). This technology enables one to enhance antigen-binding activity by introducing amino acid mutations into the complementarity determining (CDR) region of a variable region or such. The enhancement of antigen-binding ability improves in vitro biological activity or reduces dosage, and thus improves in vivo efficacy (Non Patent Literature 8). Currently, clinical trials are being conducted to assess Motavizumab (produced by affinity maturation), which is expected to have a superior effect to Palivizumab, a first-generation anti-RSV antibody pharmaceutical (Non Patent Literature 9). An anti-IL-6 receptor antibody with an affinity of about 0.05 nM (i.e., greater affinity than that of TOCILIZUMAB) has been reported (Patent Literature 4). However, there is no report describing a human, humanized, or chimeric antibody having an affinity greater than 0.05 nM.

[0007] A problem encountered with current antibody pharmaceuticals is the high production cost associated with the administration of extremely large quantities of protein. For example, the dosage of TOCILIZUMAB, a humanized anti-IL-6 receptor IgG1 antibody, has been estimated to be about 8 mg/kg/month by intravenous injection (Non Patent Literature 4). Its preferred form of administration is subcutaneous formulation in chronic autoimmune diseases. In general, it is necessary that subcutaneous formulations are high-concentration formulations. From the perspective of stability or such, the limit for IgG-type antibody formulations is generally about 100 mg/ml (Non Patent Literature 10). Low-cost, convenient second-generation antibody pharmaceuticals that can be administered subcutaneously in longer intervals can be provided by increasing the half-life of an antibody in the plasma to prolong its therapeutic effect

and thereby reduce the amount of protein administered, and by conferring the antibody with high stability.

[0008] FcRn is closely involved in antibody pharmacokinetics. With regard to differences in the plasma half-life of antibody isotypes, IgG1 and IgG2 are known to have superior plasma half-life to IgG3 and IgG4 (Non Patent Literature 11). As a method for further improving the plasma half-life of IgG1 and IgG2 antibodies which have superior plasma half-lives, substitution of amino acids in the constant region which enhances the binding to FcRn has been reported (Non Patent Literature 12 and 13). From the viewpoint of immunogenicity, the plasma half-life is further improved by substituting amino acids preferably in the variable region rather than in the constant region (Patent Literature 5). However, there is no report to date on the improvement of the plasma half-life of IL-6 receptor antibodies through alteration of the variable region.

[0009] Another important problem encountered in the development of biopharmaceuticals is immunogenicity. In general, the immunogenicity of mouse antibodies is reduced by antibody humanization. It is assumed that the immunogenicity risk can be further reduced by using a germline framework sequence as a template in antibody humanization (Non Patent Literature 14). However, even Adalimumab, a fully human anti-TNF antibody, showed high-frequency (13% to 17%) immunogenicity, and the therapeutic effect was found to be reduced in patients who showed immunogenicity (Non Patent Literature 15 and 16). T-cell epitopes may be present even in the CDR of human antibodies, and these T-cell epitopes in CDR are a possible cause of immunogenicity. In silico and in vivo methods for predicting T-cell epitopes have been reported (Non Patent Literature 17 and 18). It is assumed that immunogenicity risk can be reduced by removing T-cell epitopes using such methods (Non Patent Literature 19).

[0010] TOCILIZUMAB, a humanized anti-IL-6 receptor IgG1 antibody, is an IgG1 antibody obtained by humanizing mouse antibody PM1. CDR grafting is carried out using human NEW and REI sequences as template framework for H and L chains, respectively; however, five mouse sequence amino acids are retained in the framework as essential amino acids for maintaining the activity (Non Patent Literature 20). There is no previous report that fully humanizes the remaining mouse sequence in the framework of the humanized antibody TOCILIZUMAB without reducing the activity. Furthermore, the CDR sequence of TOCILIZUMAB is a mouse sequence, and thus, like Adalimumab, it may have T-cell epitopes in the CDR, which may have a potential immunogenicity risk. In clinical trials of TOCILIZUMAB, anti-TOCILIZUMAB antibodies were not detected at the effective dose of 8 mg/kg, but they were observed at the doses of 2 mg/kg and 4 mg/kg (Patent Literature 6). This suggests that there is still room for improvement with regard to the immunogenicity of TOCILIZUMAB.

However, there has been no report on reducing the immunogenicity risk of TOCILIZUMAB by amino acid substitution.

[0011] The isotype of TOCILIZUMAB is IgG1. The isotype difference refers to differences in the constant region sequence. Since the constant region sequence is assumed to have strong influence on the effector function, pharmacokinetics, physical properties, and so on, selection of the constant region sequence is very important for the development of antibody pharmaceuticals (Non Patent Literature 11). In recent years, the safety of antibody pharmaceuticals has become of great importance. Interaction between the antibody Fc portion and the Fc gamma receptor (effector function) may have caused serious adverse effects in phase-I clinical trials of TGN1412 (Non Patent Literature 21). For antibody pharmaceuticals designed to neutralize the biological activity of an antigen, the binding to the Fc gamma receptor, which is important for effector functions such as ADCC, is unnecessary. The binding to the Fc gamma receptor may even be unfavorable from the viewpoint of adverse effects. A method for reducing the binding to the Fc gamma receptor is to alter the isotype of an IgG antibody from IgG1 to IgG2 or IgG4 (Non Patent Literature 22). IgG2 is more favorable than IgG4 from the viewpoint of pharmacokinetics and Fc gamma receptor I binding (Non Patent Literature 11). TOCILIZUMAB is an IL-6 receptor-neutralizing antibody, and its isotype is IgG1. Thus, in view of the potential adverse effects, IgG2 may be a preferred isotype since effector functions such as ADCC are not needed.

[0012] Meanwhile, when developing antibody pharmaceuticals, physicochemical properties of the proteins, in particular, homogeneity and stability are very crucial. It has been reported that for the IgG2 isotype, there is significant heterogeneity derived from the disulfide bonds in the hinge region (Non Patent Literature 23). It is not easy and would be more costly to manufacture them as a pharmaceutical in large-scale while maintaining the objective substances/related substances-related heterogeneity derived from disulfide bonds between productions. Thus, single substances are desirable as much as possible. Furthermore, for heterogeneity of the H-chain C-terminal sequences of an antibody, deletion of C-terminal amino acid lysine residue, and amidation of the C-terminal carboxyl group due to deletion of both of the two C-terminal amino acids, glycine and lysine, have been reported (Non Patent Literature 24). In developing IgG2 isotype antibodies as pharmaceuticals, it is preferable to reduce such heterogeneity and maintain high stability. To produce convenient, stable, high-concentration, subcutaneously-administered formulations, it is preferable that not only the stability is high, but also the plasma half-life is superior to that of IgG1 which is the isotype of TOCILIZUMAB. However, there is no previous report on constant region sequences for antibodies with the IgG2-isotype constant region that have reduced heterogeneity, high stability, and superior plasma half-life than antibodies with the IgG1 isotype

constant region.

Citation List

Patent Literature

[0013] PTL 1: WO 92/19759

PTL 2: WO 96/11020

PTL 3: WO 96/12503

PTL 4: WO 2007/143168

PTL 5: WO 2007/114319

PTL 6: WO 2004/096273

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Summary of Invention

[0015] An objective of present invention is to provide pharmaceutical formulations (herein below, may also be referred to as "agents" or "pharmaceutical compositions") that comprise second-generation molecules which are superior to the humanized anti-IL-6 receptor IgG1 antibody TOCILIZUMAB, by altering the amino acid sequences of the variable and constant regions of TOCILIZUMAB to enhance the antigen-neutralizing ability and improve pharmacokinetics, such that prolonged therapeutic effect is exerted with a less frequency of administration, and immunogenicity, safety, and physico-chemical properties (stability and homogeneity) are improved.

[0016] Furthermore, the formulations of present invention provide high concentrated polypeptide and/or antibody-containing formulations, in which dimerization and deamidation during long-term storage or multiple freeze/thaw cycles are inhibited, and which are stable and preferably suitable for the use in subcutaneous administration.

[0017] Intensive studies have been conducted to solve above mentioned problem, and it has been discovered that a stable, high concentrated polypeptide and/or antibody-containing formulation can be obtained by adding the amino acid arginine or a salt thereof as a stabilizer to the solution.

[0018] Present invention will now be described in detail. The studies were focused to obtain a pharmaceutical formulation which is stable in view of second-generation molecules that are superior to the first-generation humanized anti-IL-6 receptor IgG1 antibody TOCILIZUMAB. As a result with regard to the antibody in question, the inventors discovered multiple CDR mutations in the variable regions of TOCILIZUMAB that improve the binding ability (affinity) to the antigen. Present inventors thus successfully improved the affinity significantly by using a combination of such mutations. The inventors also succeeded in improving pharmacokinetics by introducing modifications that lower the isoelectric point of the variable region sequence. The present inventors also succeeded in improving pharmacokinetics by making the binding to the IL-6 receptor antigen to be pH-dependent, so that a single antibody molecule can neutralize the antigen multiple times. Furthermore, they successfully reduced the risk of immuno-genicity by fully humanizing the mouse-derived sequences that remain in the framework of TOCILIZUMAB and reducing the number of T-cell epitope peptides in the variable regions predicted in silico. Furthermore, the inventors also successfully

discovered novel constant region sequences for the constant region of TOCILIZUMAB, that reduce the binding to the Fc gamma receptor as compared to IgG1 to improve safety, improve the pharmacokinetics as compared to IgG1, and reduce the heterogeneity due to disulfide bonds in the hinge region of IgG2 and the heterogeneity due to the H chain C-terminus without decreasing stability. The inventors successfully produced second-generation molecules that are superior to TOCILIZUMAB by appropriately combining these amino acid sequence alterations in the CDR, variable regions, and constant regions.

[0019] Present invention relates to pharmaceutical formulations comprising a humanized anti-IL-6 receptor IgG antibody having superior antigen (IL-6 receptor)-binding ability, superior pharmacokinetics, superior safety and physical properties (stability and homogeneity), and further reduced immunogenicity risk, by altering the amino acid sequences of variable and constant regions of the humanized anti-IL-6 receptor IgG1 antibody TOCILIZUMAB; and methods for producing such pharmaceutical compositions. More specifically, the present invention provides:

(1) A pharmaceutical formulation, comprising at least one polypeptide selected from:

(a) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 1 (CDR1 of VH4-M73), CDR2 comprising the sequence of SEQ ID NO: 2 (CDR2 of VH4-M73), and CDR3 comprising the sequence of SEQ ID NO: 3 (CDR3 of VH4-M73);

(b) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 4 (CDR1 of VH3-M73), CDR2 comprising the sequence of SEQ ID NO: 5 (CDR2 of VH3-M73), and CDR3 comprising the sequence of SEQ ID NO: 6 (CDR3 of VH3-M73);

(c) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 7 (CDR1 of VH5-M83), CDR2 comprising the sequence of SEQ ID NO: 8 (CDR2 of VH5-M83), and CDR3 comprising the sequence of SEQ ID NO: 9 (CDR3 of VH5-M83);

(d) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 10 (CDR1 of VL1), CDR2 comprising the sequence of SEQ ID NO: 11 (CDR2 of VL1), and CDR3 comprising the sequence of SEQ ID NO: 12 (CDR3 of VL1);

(e) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 13 (CDR1 of VL3), CDR2 comprising the sequence of SEQ ID NO: 14 (CDR2 of VL3), and CDR3 comprising the sequence of SEQ ID NO: 15 (CDR3 of VL3); and

(f) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 16 (CDR1 of VL5), CDR2 comprising the sequence of SEQ ID NO: 17 (CDR2 of VL5), and CDR3 comprising the sequence of SEQ ID NO: 18 (CDR3 of VL5).

(2) A pharmaceutical formulation, comprising at least one antibody selected from:

(a) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 1 (CDR1 of VH4-M73), CDR2 comprising the sequence of SEQ ID NO: 2 (CDR2 of VH4-M73), and CDR3 comprising the sequence of SEQ ID NO: 3 (CDR3 of VH4-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 10 (CDR1 of VL1), CDR2 comprising the sequence of SEQ ID NO: 11 (CDR2 of VL1), and CDR3 comprising the sequence of SEQ ID NO: 12 (CDR3 of VL1);
(b) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 4 (CDR1 of VH3-M73), CDR2 comprising the sequence of SEQ ID NO: 5 (CDR2 of VH3-M73), and CDR3 comprising the sequence of SEQ ID NO: 6 (CDR3 of VH3-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 13 (CDR1 of VL3), CDR2 comprising the sequence of SEQ ID NO: 14 (CDR2 of VL3), and CDR3 comprising the sequence of SEQ ID NO: 15 (CDR3 of VL3); and
(c) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 7 (CDR1 of VH5-M83), CDR2 comprising the sequence of SEQ ID NO: 8 (CDR2 of VH5-M83), and CDR3 comprising the sequence of SEQ ID NO: 9 (CDR3 of VH5-M83), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 16 (CDR1 of VL5), CDR2 comprising the sequence of SEQ ID NO: 17 (CDR2 of VL5), and CDR3 comprising the sequence of SEQ ID NO: 18 (CDR3 of VL5).
(d) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 19 (variable region of VH4-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 22 (variable region of VL1);
(e) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 20 (variable region of VH3-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 23 (variable region of VL3); and
(f) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 (variable region of VH5-M83) and a light chain variable region comprising the sequence of SEQ ID NO: 24 (variable region of VL5).
(g) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 25 (VH4-M73) and a light chain comprising the sequence of SEQ ID NO: 28 (VL1);
(h) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 26 (VH3-M73) and a light chain comprising the sequence of SEQ ID NO: 29 (VL3); and
(i) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 27 (VH5-M83) and a light chain comprising the sequence of SEQ ID NO: 30 (VL5).
(3) A stable pharmaceutical formulation according to (1) or (2), comprising a histidine

and/or citrate buffer.

(4) A stable pharmaceutical formulation according to any one of (1)-(3), comprising at least one cationic amino acid.

(5) A stable pharmaceutical formulation according to any one of (1)-(4), comprising 1-500 mM histidine and/or citrate buffer, 1-1500 mM of at least one cationic amino acid, 1-200 mg/mL antibody, and 1-400 mM of a carbohydrate.

(6) The formulation according to (4) or (5), whereby the cationic amino acid is arginine.

(7) The formulation according to (5) or (6), whereby the carbohydrate is sucrose or trehalose.

(8) The formulation according to any one of (1)-(7) further comprising a surfactant.

(9) The formulation according to any one of (1)-(8) containing the polypeptide and/or antibody in an amount of at least 10 mg/ml.

(10) The formulation according to any one of (1)-(9) containing the polypeptide and/or antibody in an amount of at least 50 mg/ml.

(11) The formulation according to any one of (1)-(10) containing the polypeptide and/or antibody in an amount of at least 80 mg/ml.

(12) The formulation according to any one of (1)-(11) containing the polypeptide and/or antibody in an amount of less than or equal to 240 mg/ml.

(13) The formulation according to any one of (1)-(12) having a pH in the range from 4.5 to 7.0.

(14) The formulation according to (13) having a pH range from 5.5 to 6.6.

(15) The formulation according to any one of (1)-(14), whereby the formulation is liquid.

(16) The formulation according to (15) which has not been subjected to lyophilization during preparation of the formulation.

(17) The formulation according to any one of (1)-(16), wherein the dimerization of the polypeptide and/or antibody molecules is reduced.

(18) The formulation according to any one of (1)-(17), wherein the dimerization of the polypeptide and/or antibody molecules is inhibited.

(19) The formulation according to any one of (1)-(18), which is for subcutaneous administration.

(20) A method for stabilizing a solution containing the antibody, comprising adding at least one cationic amino acid, wherein the antibody is at least one antibody selected from:

(a) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 1 (CDR1 of VH4-M73), CDR2 comprising the sequence of SEQ ID NO: 2 (CDR2 of VH4-M73), and CDR3 comprising the

sequence of SEQ ID NO: 3 (CDR3 of VH4-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 10 (CDR1 of VL1), CDR2 comprising the sequence of SEQ ID NO: 11 (CDR2 of VL1), and CDR3 comprising the sequence of SEQ ID NO: 12 (CDR3 of VL1);
(b) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 4 (CDR1 of VH3-M73), CDR2 comprising the sequence of SEQ ID NO: 5 (CDR2 of VH3-M73), and CDR3 comprising the sequence of SEQ ID NO: 6 (CDR3 of VH3-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 13 (CDR1 of VL3), CDR2 comprising the sequence of SEQ ID NO: 14 (CDR2 of VL3), and CDR3 comprising the sequence of SEQ ID NO: 15 (CDR3 of VL3);
(c) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 7 (CDR1 of VH5-M83), CDR2 comprising the sequence of SEQ ID NO: 8 (CDR2 of VH5-M83), and CDR3 comprising the sequence of SEQ ID NO: 9 (CDR3 of VH5-M83), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 16 (CDR1 of VL5), CDR2 comprising the sequence of SEQ ID NO: 17 (CDR2 of VL5), and CDR3 comprising the sequence of SEQ ID NO: 18 (CDR3 of VL5);
(d) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 19 (variable region of VH4-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 22 (variable region of VL1);
(e) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 20 (variable region of VH3-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 23 (variable region of VL3);
(f) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 (variable region of VH5-M83) and a light chain variable region comprising the sequence of SEQ ID NO: 24 (variable region of VL5);
(g) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 25 (VH4-M73) and a light chain comprising the sequence of SEQ ID NO: 28 (VL1);
(h) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 26 (VH3-M73) and a light chain comprising the sequence of SEQ ID NO: 29 (VL3); and
(i) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 27 (VH5-M83) and a light chain comprising the sequence of SEQ ID NO: 30 (VL5).
(21) A method for stabilizing an antibody during freeze/thaw cycles of a solution containing the antibody, comprising adding at least one cationic amino acid, wherein the antibody is at least one antibody selected from:
(a) an antibody which comprises a heavy chain variable region that comprises CDR1

comprising the sequence of SEQ ID NO: 1 (CDR1 of VH4-M73), CDR2 comprising the sequence of SEQ ID NO: 2 (CDR2 of VH4-M73), and CDR3 comprising the sequence of SEQ ID NO: 3 (CDR3 of VH4-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 10 (CDR1 of VL1), CDR2 comprising the sequence of SEQ ID NO: 11 (CDR2 of VL1), and CDR3 comprising the sequence of SEQ ID NO: 12 (CDR3 of VL1);

(b) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 4 (CDR1 of VH3-M73), CDR2 comprising the sequence of SEQ ID NO: 5 (CDR2 of VH3-M73), and CDR3 comprising the sequence of SEQ ID NO: 6 (CDR3 of VH3-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 13 (CDR1 of VL3), CDR2 comprising the sequence of SEQ ID NO: 14 (CDR2 of VL3), and CDR3 comprising the sequence of SEQ ID NO: 15 (CDR3 of VL3);

(c) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 7 (CDR1 of VH5-M83), CDR2 comprising the sequence of SEQ ID NO: 8 (CDR2 of VH5-M83), and CDR3 comprising the sequence of SEQ ID NO: 9 (CDR3 of VH5-M83), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 16 (CDR1 of VL5), CDR2 comprising the sequence of SEQ ID NO: 17 (CDR2 of VL5), and CDR3 comprising the sequence of SEQ ID NO: 18 (CDR3 of VL5);

(d) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 19 (variable region of VH4-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 22 (variable region of VL1);

(e) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 20 (variable region of VH3-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 23 (variable region of VL3);

(f) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 (variable region of VH5-M83) and a light chain variable region comprising the sequence of SEQ ID NO: 24 (variable region of VL5);

(g) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 25 (VH4-M73) and a light chain comprising the sequence of SEQ ID NO: 28 (VL1);

(h) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 26 (VH3-M73) and a light chain comprising the sequence of SEQ ID NO: 29 (VL3); and

(i) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 27 (VH5-M83) and a light chain comprising the sequence of SEQ ID NO: 30 (VL5).

[0020] The above described humanized anti-IL-6 receptor IgG antibodies have enhanced efficacy and improved pharmacokinetics; thus, they can exert a prolonged therapeutic

effect with a less administration frequency.

Brief Description of Drawings

[0021] [fig.1]Fig. 1 is a listing of mutation sites that improve the affinity of TOCILIZUMAB for the IL-6 receptor. The HCDR2 sequence of TOCILIZUMAB is shown in SEQ ID NO: 81; the HCDR2 sequence after mutation (upper line) is shown in SEQ ID NO: 82; the HCDR2 sequence after mutation (lower line) is shown in SEQ ID NO: 83; the HCDR3 sequence of TOCILIZUMAB is shown in SEQ ID NO: 84; the HCDR3 sequence after mutation (upper line) is shown in SEQ ID NO: 85; the HCDR3 sequence after mutation (lower line) is shown in SEQ ID NO: 86; the LCDR1 sequence of TOCILIZUMAB is shown in SEQ ID NO: 87; the LCDR1 sequence after mutation (upper line) is shown in SEQ ID NO: 88; the LCDR1 sequence after mutation (lower line) is shown in SEQ ID NO: 89; the LCDR3 sequence of TOCILIZUMAB is shown in SEQ ID NO: 90; the LCDR3 sequence after mutation (upper line) is shown in SEQ ID NO: 91; and the LCDR3 sequence after mutation (lower line) is shown in SEQ ID NO: 92.

[fig.2]Fig. 2 is a graph showing the neutralizing activities of TOCILIZUMAB and RDC-23 in BaF/gp130.

[fig.3]Fig. 3 is a listing of mutation sites that can reduce the isoelectric point of variable region without significantly reducing the binding of TOCILIZUMAB to the IL-6 receptor. Asterisk in the drawing represents a site that has no influence on the iso-electric point but which was mutated for conversion into a human sequence. The HFR1 sequence of TOCILIZUMAB is shown in SEQ ID NO: 93; the HFR1 sequence after mutation is shown in SEQ ID NO: 94; the HCDR1 sequence of TOCILIZUMAB is shown in SEQ ID NO: 95; the HCDR1 sequence after mutation is shown in SEQ ID NO: 96; the HFR2 sequence of TOCILIZUMAB is shown in SEQ ID NO: 97; the HFR2 sequence after mutation is shown in SEQ ID NO: 98; the HCDR2 sequence of TOCILIZUMAB is shown in SEQ ID NO: 81; the HCDR2 sequence after mutation is shown in SEQ ID NO: 99; the HFR4 sequence of TOCILIZUMAB is shown in SEQ ID NO: 100; the HFR4 sequence after mutation is shown in SEQ ID NO: 101; the LFR1 sequence of TOCILIZUMAB is shown in SEQ ID NO: 102; the LFR1 sequence after mutation is shown in SEQ ID NO: 103; the LCDR1 sequence of TOCILIZUMAB is shown in SEQ ID NO: 87; the LCDR1 sequence after mutation is shown in SEQ ID NO: 104; the LFR2 sequence of TOCILIZUMAB is shown in SEQ ID NO: 105; the LFR2 sequence after mutation is shown in SEQ ID NO: 106; the LCDR2 sequence of TOCILIZUMAB is shown in SEQ ID NO: 107; the LCDR2 sequences after mutation are shown in SEQ ID Nos: 108 and 109; the LFR3 sequence of TOCILIZUMAB is shown in SEQ ID NO: 110; the LFR3 sequence after mutation is shown in SEQ ID

NO: 111; the LFR4 sequence of TOCILIZUMAB is shown in SEQ ID NO: 112; and the LFR4 sequence after mutation is shown in SEQ ID NO: 113.

[fig.4]Fig. 4 is a graph showing the neutralizing activities of TOCILIZUMAB and H53/L28 in BaF/gp130.

[fig.5]Fig. 5 is a graph showing the time courses of plasma concentration for TOCILIZUMAB and H53/L28 in mice after intravenous administration.

[fig.6]Fig. 6 is a graph showing the time courses of plasma concentration for TOCILIZUMAB and H53/L28 in mice after subcutaneous administration.

[fig.7]Fig. 7 is a schematic illustration showing that an IgG molecule can bind again to another antigen by dissociating from a membrane-type antigen in the endosome.

[fig.8]Fig. 8 is a listing of mutation sites that can confer pH dependency to the binding of TOCILIZUMAB to the IL-6 receptor (binding at pH 7.4 and dissociation at pH 5.8). The HFR1 sequence of TOCILIZUMAB is shown in SEQ ID NO: 93; the HFR1 sequence after mutation is shown in SEQ ID NO: 114; the HCDR1 sequence of TOCILIZUMAB is shown in SEQ ID NO: 95; the HCDR1 sequence after mutation is shown in SEQ ID NO: 115; the LCDR1 sequence of TOCILIZUMAB is shown in SEQ ID NO: 87; the LCDR1 sequence after mutation is shown in SEQ ID NO: 116; the LCDR2 sequence of TOCILIZUMAB is shown in SEQ ID NO: 107; and the LCDR2 sequence after mutation is shown in SEQ ID NO: 117.

[fig.9]Fig. 9 is a graph showing the neutralizing activities of TOCILIZUMAB and H3pI/L73 in BaF/gp130.

[fig.10]Fig. 10 is a graph showing the time courses of plasma concentration for TOCILIZUMAB and H3pI/L73 in cynomolgus monkeys after intravenous administration.

[fig.11]Fig. 11 is a graph showing the time courses of plasma concentration for TOCILIZUMAB and H3pI/L73 in human IL-6 receptor transgenic mice after intravenous administration.

[fig.12]Fig. 12 is a diagram showing the result of assessment of the C-terminus-derived heterogeneity of TOCILIZUMAB, TOCILIZUMAB delta K, and TOCILIZUMAB delta GK by cation exchange chromatography.

[fig.13]Fig. 13 is a diagram showing the result of assessment of the disulfide bond-derived heterogeneity of TOCILIZUMAB-IgG1, TOCILIZUMAB-IgG2, and TOCILIZUMAB-SKSC by cation exchange chromatography.

[fig.14]Fig. 14 is a diagram showing the denaturation curves for TOCILIZUMAB-IgG1, TOCILIZUMAB-IgG2, and TOCILIZUMAB-SKSC obtained by differential scanning calorimetry (DSC), and the Tm value for each Fab domain.

[fig.15]Fig. 15 is a graph showing the time courses of plasma concentration for TOCILIZUMAB-IgG1, TOCILIZUMAB-M44, TOCILIZUMAB-M58, and

TOCILIZUMAB-M73 in human FcRn transgenic mice after intravenous administration.

[fig.16]Fig. 16 is a graph showing the neutralizing activities of TOCILIZUMAB, control, and Fv5-M83 in BaF/gp130.

[fig.17]Fig. 17 is a graph showing the neutralizing activities of TOCILIZUMAB, Fv3-M73, and Fv4-M73 in BaF/gp130.

[fig.18]Fig. 18 is a graph showing the time courses of plasma concentrations for TOCILIZUMAB, control, Fv3-M73, Fv4-M73, and Fv5-M83 in cynomolgus monkeys after intravenous administration.

[fig.19]Fig. 19 is a graph showing the time courses of CRP concentration for TOCILIZUMAB, control, Fv3-M73, Fv4-M73, or Fv5-M83 in cynomolgus monkeys after intravenous administration.

[fig.20]Fig. 20 is a graph showing the time courses of percentage of free soluble IL-6 receptor in cynomolgus monkeys after intravenous administration of TOCILIZUMAB, control, Fv3-M73, Fv4-M73, or Fv5-M83.

[fig.21]Fig. 21 is a graph showing the differences in the formation of high molecular weight species (HMW) of the antibody Fv4-M73 over time in for different formulations (A-D).

[fig.22]Fig. 22 is a graph showing the differences in the formation of high molecular weight species (delta HMW: increase from initial) of the antibody Fv4-M73 at two points in time (at 25 degrees C) with different pH values, buffer species, NaCl concentrations and with or without arginine.

[fig.23]Fig. 23 is a graph showing the differences in the formation of high molecular weight species (HMW) of the antibody Fv4-M73 at one point in time (at 25 degrees C) with different pH values, buffer species, NaCl concentrations and with or without arginine.

[fig.24]Fig. 24 is a graph showing the differences in the formation of high molecular weight species (delta HMW: increase from initial) of the antibody Fv4-M73 at three different points in time (at 40 degrees C) with different pH values, buffer species, NaCl concentrations and with or without arginine.

[fig.25]Fig. 25 is a graph showing the differences in the formation of high molecular weight species (HMW) of the antibody Fv4-M73 at one point in time (at 40 degrees C) with different pH values, buffer species, NaCl concentrations and with or without arginine.

[fig.26]Fig. 26 is a graph showing the differences in the formation of low molecular weight species (delta LMW: increase from initial) of the antibody Fv4-M73 at three different points in time (at 40 degrees C) with different pH values, buffer species, NaCl concentrations and with or without arginine.

[fig.27]Fig. 27 is a diagram showing the results of an anion exchange chromatography carried out with solutions of the antibody Fv4-M73 stored under different pH values.
[fig.28]Fig. 28 are diagrams showing the results of an anion exchange chromatography carried out with solutions of the antibody Fv4-M73 stored under three different pH values, different concentrations of NaCl, arginine and two different buffer species.
[fig.29]Fig. 29 is a graph showing the differences in the formation of high molecular weight species (delta HMW: increase from initial) of the antibody Fv4-M73 for two different numbers of freeze/thaw cycles, different concentrations of NaCl, arginine and two different buffer species.
[fig.30]Fig. 30 is a graph showing the differences in the formation of high molecular weight species (delta HMW: increase from initial) of the antibody Fv4-M73 at three different points in time (at 40 degrees C and 25 degrees C) and four different formulations (E-H).
[fig.31]Fig. 31 is a graph showing the differences in the formation of high molecular weight species (delta HMW: increase from initial) of the antibody Fv4-M73 for two different numbers of freeze/thaw cycles and four different formulations (E-H).
[fig.32]Fig.32 is a graph showing the differences in the formation of high molecular weight species (delta HMW: increase from initial) of the antibody Fv4-M73 for six different formulations after three months and six months at 5 degrees C.
[fig.33]Fig.33 is a graph showing the differences in the formation of high molecular weight species (delta HMW: increase from initial) of the antibody Fv4-M73 for six different formulations after three months and six months at -20 degrees C.

Description of Embodiments

[0022] The present invention provides a pharmaceutical formulation, comprising at least one polypeptide selected from:

(a) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 1 (CDR1 of VH4-M73), CDR2 comprising the sequence of SEQ ID NO: 2 (CDR2 of VH4-M73), and CDR3 comprising the sequence of SEQ ID NO: 3 (CDR3 of VH4-M73);

(b) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 4 (CDR1 of VH3-M73), CDR2 comprising the sequence of SEQ ID NO: 5 (CDR2 of VH3-M73), and CDR3 comprising the sequence of SEQ ID NO: 6 (CDR3 of VH3-M73);

(c) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 7 (CDR1 of VH5-M83), CDR2 comprising the sequence of SEQ ID NO: 8 (CDR2 of VH5-M83), and CDR3 comprising the sequence of SEQ ID NO: 9 (CDR3 of VH5-M83);

(d) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 10 (CDR1 of VL1), CDR2 comprising the sequence of SEQ ID NO: 11 (CDR2 of VL1), and CDR3 comprising the sequence of SEQ ID NO: 12 (CDR3 of VL1);
(e) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 13 (CDR1 of VL3), CDR2 comprising the sequence of SEQ ID NO: 14 (CDR2 of VL3), and CDR3 comprising the sequence of SEQ ID NO: 15 (CDR3 of VL3); and
(f) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 16 (CDR1 of VL5), CDR2 comprising the sequence of SEQ ID NO: 17 (CDR2 of VL5), and CDR3 comprising the sequence of SEQ ID NO: 18 (CDR3 of VL5).
The polypeptides and antibodies of present invention can be formulated according to conventional methods (see, for example, Remington's Pharmaceutical Science, latest edition, Mark Publishing Company, Easton, USA).

[0023] "Pharmaceutical formulation", "agent", and "pharmaceutical composition" as used in this inventions means a liquid or solid formulation containing polypeptides and/or antibodies as active component(s), which is prepared so that it is suitable for administration to an animal such as human directly or after reconstitution. If needed, the formulation may contain pharmaceutically acceptable carriers and/or additives. For example, detergents (e.g. PEG, Tween, Pluronic), excipients, antioxidants (e.g. ascorbic acid, methionine), coloring agents, flavoring agents, preservatives, stabilizers, buffering agents, chelating agents (e.g. EDTA), suspending agents, isotonizing agents, binders, disintegrants, lubricants, fluidity promoters, and corrigents.

[0024] The polypeptide and/or antibody-containing formulation according to present invention preferably does not contain HSA (human serum albumin), gelatin, and such proteins as stabilizing agent.

[0025] The polypeptide and/or antibody-containing formulation according to present invention is preferably a liquid pharmaceutical formulation containing a polypeptide and/or an antibody in a high concentration of not less than 10 mg/mL, preferably not less than 50 mg/mL, and more preferably not less than 80 mg/mL. The concentration is from 10 to 240 mg/mL, and may be 10 mg/mL, or more preferably, 50 mg/mL, and still more preferably, from 100 to 200 mg/mL.

[0026] Liquid pharmaceutical formulation according to present invention is preferably produced by not conducting lyophilization step(s).

[0027] A buffering agent which can be used in present invention is one which can adjust the pH in the desired range and which is pharmaceutically acceptable. In a high concentration polypeptide and/or antibody-containing formulation according to present invention, the pH of the formulation is preferably 4.5 to 7, and more preferred 5.5 to 6.6. Those buffering agents are known by those skilled in the art, and examples thereof include inorganic salts such as phosphoric acid salts (sodium or potassium) and sodium

hydrogen carbonate; organic acid salts such as citric acid salts (sodium or potassium), sodium acetate and sodium succinate; and acids such as phosphoric acid, carbonic acid, citric acid, succinic acid, malic acid and gluconic acid. Furthermore, Tris buffers, Good's buffers such as MES and MOPS, histidine (e.g., histidine hydrochloric acid salt) and glycine can also be used. In the high concentration polypeptide and/or antibody-containing formulation according to present invention, the buffer is preferably a histidine or citrate buffer, whereas a histidine buffer is especially preferred. The concentration of the buffer solution is generally 1 to 500 mM, preferably 5 to 100 mM, more preferably 10 to 20 mM. In cases where a histidine buffer is used, the buffer solution contains histidine at a concentration of preferably 5 to 25 mM, more preferably 10 to 20 mM.

[0028] The formulation according to present invention can further contain a surfactant. Typical examples of surfactants include nonionic surfactants, for example, sorbitan fatty acid esters such as sorbitan monocaprylate, sorbitan monolaurate and sorbitan monopalmitate; glycerin fatty acid esters such as glycerol monocaprylate, glycerol monomyristate and glycerol monostearate; polyglycerol fatty acid esters such as decaglyceryl monostearate, decaglyceryl distearate and decaglyceryl monolinoleate; polyoxyethylene sorbitan fatty acid esters such as polyoxyethylene sorbitan monolaurate, polyoxyethylene sorbitan monooleate, polyoxyethylenesorbitan monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene sorbitan trioleate and polyoxyethylene sorbitan tristearate; polyoxyethylene sorbitol fatty acid esters such as polyoxyethylene sorbitol tetrastearate and polyoxyethylene sorbitol tetra oleate; polyoxyethylene glycerin fatty acid esters such as polyoxyethylene glyceryl monostearate; polyethylene glycol fatty acid esters such as polyethylene glycol distearate; polyoxyethylene alkyl ethers such as polyoxyethylene lauryl ether; polyoxyethylene polyoxypropylene alkyl ethers such as polyoxyethylene polyoxypropylene glycol ether, polyoxyethylene polyoxypropylene propyl ether and polyoxyethylene polyoxypropylene cetyl ether; polyoxyethylene alkyl phenyl ethers such as polyoxyethylene nonylphenyl ether; polyoxyethylene hardened castor oils such as polyoxyethylene castor oil and polyoxyethylene hardened castor oil (polyoxyethylene hydrogenated castor oil); polyoxyethylene bees wax derivatives such as polyoxyethylene sorbitol bees wax; polyoxyethylene lanolin derivatives such as polyoxyethylene lanolin; surfactants having an HLB of 6 to 18 such as polyoxyethylene fatty acid amides, for example, polyoxyethylene octadecanamide; anionic surfactants, for example, alkyl sulfate salts having a C_{10}-C_{18} alkyl group, such as sodium cetyl sulfate, sodium lauryl sulfate and sodium oleyl sulfate; polyoxyethylene alkyl ether sulfate salts in which the average number of moles of the added ethylene oxide units is 2 to 4 and the number of carbon atoms of the alkyl group is 10 to 18, such as poly-

oxyethylene sodium lauryl sulfate; alkyl sulfosuccinate salts having a C_8-C_{18} alkyl group, such as sodium lauryl sulfosuccinate; natural surfactants such as lecithin and glycerophospholipids; sphingophospholipids such as sphingomyelin; and sucrose esters of C_{12}-C_{18} fatty acids. These surfactants can be added to the formulation of the present invention individually, or two or more of these surfactants can be added in combination.

[0029] Preferred surfactants are polyoxyethylene sorbitan fatty acid esters and polyoxyethylene polyoxypropylene alkyl ethers, and especially preferred are polysorbates 20, 21, 40, 60, 65, 80, 81 and 85, and Pluronic type surfactants, and most preferred are polysorbates 20 and 80, and Pluronic F-68 (Poloxamer 188).

[0030] The amount of the surfactant(s) to be added to the antibody formulation according to present invention is generally 0.0001 to 10% (w/v), preferably 0.001 to 5%, more preferably 0.005 to 3%.

[0031] The formulation according to present invention can further contain an acidic amino acid such as arginine, as well as methionine, glycine, alanine, phenylalanine, tryptophan, serine, threonine, asparagine, glutamine, and such amino acid as a stabilizing agent.

[0032] In antibody-containing formulations or antibody-containing solution formulations according to present invention, the formation of dimers during freeze/thaw cycles can be inhibited by adding carbohydrate(s) (e.g. sugar(s)). The sugars that can be used include nonreducing oligosaccharides, for example, nonreducing disaccharides such as sucrose and trehalose or nonreducing trisaccharides such as raffinose, and especially preferred are nonreducing oligosaccharides. Preferred nonreducing oligosaccharides are nonreducing disaccharides, more preferably sucrose and trehalose.

[0033] In antibody-containing formulations or antibody-containing solution formulations according to present invention, the formation of multimers and degradation products during long-term storage can be inhibited by adding carbohydrate(s) (e.g. sugar(s)). The sugars that can be used include sugar alcohols such as mannitol and sorbitol; and nonreducing oligosaccharides, for example, nonreducing disaccharides such as sucrose and trehalose or nonreducing trisaccharides such as raffinose, among which nonreducing oligosaccharides are especially preferred. Preferred nonreducing oligosaccharides are nonreducing disaccharides, more preferably sucrose and trehalose.

[0034] The sugars should be added at 0.1-500 mg/mL, preferably 10-300 mg/mL, more preferably 25-100 mg/mL.

[0035] In another aspect, the formulation according to present invention is preferably substantially composed of the following components:

A)modified anti-IL-6 receptor antibody;

B)cationic amino acid (e.g. arginine, histidine, and/or lysine);

C)buffering agent(s) (e.g. histidine or citrate).

[0036] Carbohydrate(s) (e.g. sugars) and/or surfactant(s) may also be included in the formulation according to purposes.

[0037] As a stabilizing agent, methionine, glycine, alanine, phenylalanine, tryptophan, serine, threonine, asparagine, glutamine, and such amino acid may be included.

[0038] The term "substantially composed of" herein means that a component other than the components usually added to formulations is not contained, the components usually added to formulations being the optional additive components described below, such as suspending agents, solubilizing agents, isotonic agents, preservatives, adsorption inhibitors, diluents, vehicles, pH-adjusters, soothing agents, sulfur-containing reducing agents and antioxidants.

[0039] Examples of the suspending agent include methyl cellulose, polysorbate 80, hydroxyethyl cellulose, gum rabic, powdered tragacanth, sodium carboxymethylcellulose and polyoxyethylene sorbitan monolaurate.

[0040] Examples of the solubilizing agent include polyoxyethylene hydrogenated castor oil, polysorbate 80, nicotinamide, polyoxyethylene sorbitan monolaurate, macrogol and castor oil fatty acid ethyl ester.

[0041] Examples of the isotonic agent include sodium chloride, potassium chloride and calcium chloride.

[0042] Examples of the preservative include methyl p-hydroxybenzoate, ethyl p-hydroxybenzoate, sorbic acid, phenol, cresol and chlorocresol.

[0043] Examples of the adsorption inhibitor include human serum albumin, lecithin, dextran, ethyleneoxide-propylene oxide copolymer, hydroxypropylcellulose, methyl cellulose, polyoxyethylene hydrogenated castor oil and polyethylene glycol.

[0044] Examples of the sulfur-containing reducing agent include the compounds having a sulfhydryl group(s), such as N-acetylcysteine, N-acetyl homocysteine, thioctic acid, thiodiglycol, thioethanolamine, thioglycerol, thiosorbitol, thioglycolic acid and salts thereof, sodium thiosulfate, glutathione and C_1-C_7 thioalkanes.

[0045] Examples of the antioxidant include erythorbic acid, dibutylhydroxytoluene, butylated hydroxyanisole, alpha-tocopherol, tocopherol acetate, L-ascorbic acid and salts thereof, L-ascorbyl palmitate, L-ascorbyl stearate, sodium hydrogen sulfite, sodium sulfite, triamyl gallate, propyl gallate, and chelating agents such as disodium ethylenediaminetetraacetate (EDTA), sodium pyrophosphate and sodium metaphosphate.

[0046] However, the formulation (agent, pharmaceutical composition) according to present invention, which can be used for preventing or treating IL-6-associated diseases including inflammatory diseases, is not limited to the above and may appropriately contain other conventional carriers. Specifically, examples include light anhydrous

silicic acid, lactose, crystalline cellulose, mannitol, starch, carmellose calcium, carmellose sodium, hydroxypropylcellulose, hydroxypropyl methylcellulose, polyvinyl acetal diethylaminoacetate, polyvinylpyrrolidone, gelatin, medium chain fatty acid triglyceride, polyoxyethylene hydrogenated castor oil 60, saccharose, carboxymethylcellulose, corn starch, and inorganic salts. They may also contain other low-molecular-weight polypeptides; proteins such as serum albumin, gelatin, and immunoglobulin; and amino acids. When preparing aqueous solutions for injection, the anti-IL-6 receptor antibodies are dissolved, for example, in isotonic solutions containing physiological saline, glucose, or other adjuvants. Adjuvants include, for example, D-sorbitol, D-mannose, D-mannitol, and sodium chloride. Furthermore, appropriate solubilizing agents, for example, alcohol (ethanol, and the like), polyalcohol (propylene glycol, PEG, and the like), and non-ionic surfactants (polysorbate 80 and HCO-50) may be combined.

[0047] If necessary, the polypeptides may be encapsulated in microcapsules (microcapsules made of hydroxycellulose, gelatin, poly(methyl methacrylate), and the like), or made into a colloidal drug delivery system (liposomes, albumin microspheres, microemulsions, nanoparticles, nanocapsules, etc) (see, for example, "Remington's Pharmaceutical Science 16th edition", Oslo Ed. (1980)). Moreover, methods for preparing agents as sustained-release agents are known, and these can be applied to the polypeptides (Langer et al., J. Biomed. Mater. Res. (1981) 15: 167-277; Langer, Chem. Tech. (1982) 12: 98-105; US Patent No. 3,773,919; European Patent Application (EP) No. 58,481; Sidman et al., Biopolymers (1983) 22:547-56; EP No.133,988). Furthermore, liquid volume for subcutaneous administration can be increased by adding or mixing hyaluronidase to an agent (for example, see WO 2004/078140).

[0048] The pharmaceutical compositions of present invention can be administered both orally and parenterally, but are preferably administered parenterally. Specifically, the compositions are administered to patients by injection or transdermally. Injections include, for example, systemic and local administrations by intravenous, intramuscular, or subcutaneous injection, or such. The compositions may be locally injected at the site of treatment or in the periphery of the site by intramuscular injection, in particular. Transdermal dosage forms include, for example, ointments, gel, cream, poultices, and patches, which can be administered locally or systemically. Furthermore, administration methods can be appropriately selected according to the patient's age and symptoms. The administered dose can be selected, for example, from the range of 0.0001 mg to 100 mg active ingredient per kg of body weight for each administration. Alternatively, when the compositions are administered to human patients, for example, the active ingredient can be selected from the range of 0.001 to 1000 mg per kg body weight for each patient. A single administration dose preferably contains, for example,

an antibody of the present invention at about 0.01 to 50 mg/kg body weight. However, the dose of an antibody of the present invention is not limited to these doses.

[0049] As can be seen from the results of the examples described below, according to present invention, a stable formulation can be obtained, in which dimerization and deamidation of the antibody during long-term storage or freezing/thawing are low, by adding one or more cationic amino acids (arginine, histidine, and/or lysine, preferably arginine), or cationic amino acid in combination with another amino acid, to the formulation.

[0050] To evaluate the shelf life stability of the high concentration antibody-containing formulation, present inventors studied the effects of various additives by conducting size exclusion chromatography and anion exchange chromatography tests. As a result, it has been found that in solutions with a high concentration of antibodies dissolved in a buffer solution containing the amino acid arginine, the amount of dimers was lower than in solutions without additional arginine. These results show, that arginine is effective as a stabilizer to inhibit antibody dimerization.

[0051] Thus, by adding arginine as a stabilizer, a stable antibody formulation can be provided, in which dimerization of the antibody is reduced.

[0052] One embodiment of the invention is a stable antibody-containing formulation, characterized by containing an antibody and arginine in a buffered solution.

[0053] As arginine used in present invention, any arginine compound per se, derivatives thereof and salts thereof can be used. L-arginine and salts thereof are preferred.

[0054] In cases where the formulation according to present invention contains arginine, the concentration of arginine is preferably 1 to 1500 mM, more preferably 50 to 1000 mM, more preferably 50 to 200 mM.

[0055] The polypeptides of present invention are not particularly limited; however, they are preferably antigen-binding substances having the activity of binding to human IL-6 receptor. Such antigen-binding substances preferably include, for example, antibody heavy chain variable regions (VH), antibody light chain variable regions (VL), antibody heavy chains, antibody light chains, and antibodies.

[0056] Of the polypeptides of (a) to (f) above, the polypeptides of (a) to (c) are preferable examples of antibody heavy chain variable regions, while the polypeptides of (d) to (f) preferable examples of antibody light chain variable regions.

[0057] These variable regions can be used as a portion of an anti-human IL-6 receptor antibody. Anti-human IL-6 receptor antibodies in which such a variable region is used have superior binding activity, excellent pharmacokinetics, excellent safety, reduced immunogenicity, and/or superior physicochemical properties. In present invention, excellent pharmacokinetics or improvement of pharmacokinetics refers to any one of: decrease in "clearance (CL)", increase in the "area under the curve (AUC)", increase in

"mean residence time", and increase in "plasma half-life (t1/2)", which are pharmacokinetic parameters calculated from the time course of plasma concentration when an antibody is administered into the body. Herein, superior physicochemical property or improved physicochemical property refers to, but is not limited to, improved stability, decreased heterogeneity, or the like.

[0058] Human antibody framework regions (FRs) to be linked with CDR are selected so that the CDR forms a favorable antigen-binding site. FRs to be used for the variable regions of the present invention are not particularly limited and any FR may be used; however, human-derived FRs are preferably used. It is possible to use human-derived FRs having a natural sequence. Alternatively, if needed, substitution, deletion, addition and/or insertion or such of one or more amino acids may be introduced into the framework region having a natural sequence so that the CDR forms an adequate antigen-binding site. Mutant FR sequences having a desired property can be selected, for example, by measuring and evaluating the binding activity to an antigen for an antibody with an FR with amino acid substitutions (Sato, K. et al., Cancer Res. (1993) 53, 851-856).

[0059] Moreover, one or more amino acids may be substituted, deleted, added, and/or inserted in the CDR sequence described above. It is preferred that a CDR sequence after substitution, deletion, addition, and/or insertion of one or more amino acids has equivalent activity to the CDR sequence before alteration with regard to binding activity, neutralizing activity, stability, immunogenicity, and/or pharmacokinetics. The number of amino acids to be substituted, deleted, added, and/or inserted is not particularly limited; however, it is preferably three amino acids or less, more preferably two amino acids or less, and still more preferably one amino acid per CDR.

[0060] Methods for substituting one or more amino acid residues with other amino acids of interest include, for example, site-directed mutagenesis (Hashimoto-Gotoh, T, Mizuno, T, Ogasahara, Y, and Nakagawa, M. (1995) An oligodeoxyribonucleotide-directed dual amber method for site-directed mutagenesis. Gene 152, 271-275; Zoller, MJ, and Smith, M. (1983) Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. 100, 468-500; Kramer, W, Drutsa, V, Jansen, HW, Kramer, B, Pflugfelder, M, and Fritz, HJ (1984) The gapped duplex DNA approach to oligonucleotide-directed mutation construction. Nucleic Acids Res. 12, 9441-9456; Kramer W, and Fritz HJ (1987) Oligonucleotide-directed construction of mutations via gapped duplex DNA Methods. Enzymol. 154, 350-367; Kunkel, TA (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci U. S. A. 82, 488-492). This method can be used to substitute desired amino acids in an antibody with other amino acids of interest. Furthermore, as a method for amino acid substitution, amino acids can be substituted to obtain appropriate frameworks and

CDRs using library techniques such as framework shuffling (Mol. Immunol. 2007 Apr; 44(11): 3049-60) and CDR repair (US 2006/0122377).

[0061] The present invention also provides a pharmaceutical formulation, comprising at least one antibody selected from:

(a) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 1 (CDR1 of VH4-M73), CDR2 comprising the sequence of SEQ ID NO: 2 (CDR2 of VH4-M73), and CDR3 comprising the sequence of SEQ ID NO: 3 (CDR3 of VH4-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 10 (CDR1 of VL1), CDR2 comprising the sequence of SEQ ID NO: 11 (CDR2 of VL1), and CDR3 comprising the sequence of SEQ ID NO: 12 (CDR3 of VL1);

(b) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 4 (CDR1 of VH3-M73), CDR2 comprising the sequence of SEQ ID NO: 5 (CDR2 of VH3-M73), and CDR3 comprising the sequence of SEQ ID NO: 6 (CDR3 of VH3-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 13 (CDR1 of VL3), CDR2 comprising the sequence of SEQ ID NO: 14 (CDR2 of VL3), and CDR3 comprising the sequence of SEQ ID NO: 15 (CDR3 of VL3); and

(c) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 7 (CDR1 of VH5-M83), CDR2 comprising the sequence of SEQ ID NO: 8 (CDR2 of VH5-M83), and CDR3 comprising the sequence of SEQ ID NO: 9 (CDR3 of VH5-M83), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 16 (CDR1 of VL5), CDR2 comprising the sequence of SEQ ID NO: 17 (CDR2 of VL5), and CDR3 comprising the sequence of SEQ ID NO: 18 (CDR3 of VL5).

(d) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 19 (variable region of VH4-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 22 (variable region of VL1);

(e) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 20 (variable region of VH3-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 23 (variable region of VL3); and

(f) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 (variable region of VH5-M83) and a light chain variable region comprising the sequence of SEQ ID NO: 24 (variable region of VL5).

(g) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 25 (VH4-M73) and a light chain comprising the sequence of SEQ ID NO: 28 (VL1);

(h) an antibody that comprises a heavy chain comprising the sequence of SEQ ID

NO: 26 (VH3-M73) and a light chain comprising the sequence of SEQ ID NO: 29 (VL3); and

(i) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 27 (VH5-M83) and a light chain comprising the sequence of SEQ ID NO: 30 (VL5).

[0062] The antibodies described above can be used as anti-human IL-6 receptor antibodies having superior binding activity, excellent pharmacokinetics, excellent safety, reduced immunogenicity, and/or superior physicochemical properties.

[0063] Human antibody framework regions to be linked with CDR of present invention are selected so that the CDR forms a favorable antigen-binding site. FRs to be used for the variable regions of present invention are not particularly limited, and any FR may be used; however, a human-derived FR is preferably used. It is possible to use human-derived FRs having a natural sequence. Alternatively, if needed, substitution, deletion, addition and/or insertion or such of one or more amino acids may be introduced into the framework region having a natural sequence so that the CDR forms an adequate antigen-binding site. Mutant FR sequences having a desired property can be selected, for example, by measuring and evaluating the binding activity to an antigen for an antibody having an FR with amino acid substitutions (Sato, K. et al., Cancer Res. (1993) 53, 851-856).

[0064] Meanwhile, the constant region to be used for an antibody in present invention is not particularly limited, and any constant region may be used. Preferred constant regions to be used for the antibodies in present invention include, for example, human-derived constant regions (constant regions derived from IgG1, IgG2, IgG3, IgG4, C kappa, C lambda, and such). One or more amino acids may be substituted, deleted, added, and/or inserted in the human-derived constant regions. The preferred human-derived heavy chain constant regions include, for example, constant regions comprising the amino acid sequence of SEQ ID NO: 31 (constant region of VH4-M73), constant regions comprising the amino acid sequence of SEQ ID NO: 32 (constant region VH3-M73)), and constant regions comprising the amino acid sequence of SEQ ID NO: 33 (constant region of VH5-M83), while the preferred human-derived light chain constant regions include, for example, constant regions comprising the amino acid sequence of SEQ ID NO: 34 (VL1), constant regions comprising the amino acid sequence of SEQ ID NO: 35 (VL3), and constant regions comprising the amino acid sequence of SEQ ID NO: 36 (VL5).

[0065] Moreover, one or more amino acids may be substituted, deleted, added, and/or inserted in the CDR sequence described above. It is preferred that a CDR sequence after substitution, deletion, addition, and/or insertion of one or more amino acids has equivalent activity to the CDR sequence before alteration with regard to binding activity, neutralizing activity, stability, immunogenicity, and/or pharmacokinetics. The

number of amino acids to be substituted, deleted, added, and/or inserted is not particularly limited; however, it is preferably three amino acids or less, more preferably two amino acids or less, and still more preferably one amino acid per CDR.

[0066] The respective variable regions of these antibodies can be used as part of a molecule reacting with the anti-human IL-6 receptor. Those variable regions may also comprise substitutions, deletions, additions, and/or insertions of one or more amino acids (for example, five amino acids or less, preferably three amino acids or less). Methods for substituting one or more amino acid residues with other amino acids of interest include, for example, the methods described above.

[0067] Present invention also includes polypeptides comprising the variable regions described above.

[0068] The respective heavy chains and light chains of these antibodies can be used as part of a molecule reacting with the anti-human IL-6 receptor. Anti-human IL-6 receptor antibodies in which these heavy chains and light chains are used have superior binding activity, excellent pharmacokinetics, excellent safety, reduced immunogenicity, and/or superior physicochemical properties.

[0069] Those heavy chains and light chains may also comprise substitutions, deletions, additions, and/or insertions of one or more amino acids (for example, ten amino acids or less, preferably five amino acids or less, and more preferably three amino acids or less). Methods for substituting one or more amino acid residues with other amino acids of interest include, for example, the methods described above.

[0070] Substitutions, deletions, additions, and/or insertions of one or more amino acids may be carried out for the variable regions, constant regions, or both.

[0071] Present invention also includes polypeptides comprising the heavy and light chains described above.

[0072] The antibodies in present invention are preferably humanized antibodies.

[0073] Humanized antibodies are also referred to as reshaped human antibodies. Such a humanized antibody is obtained by grafting a complementary determining region (CDR) derived from a non-human mammal into the CDR of a human antibody. Conventional genetic recombination techniques for the preparation of such antibodies are also known (see European Patent Application No. EP 125023; and WO 96/02576).

[0074] Specifically, for example, a DNA sequence designed such that a CDR of interest and a framework region (FR) of interest are linked is synthesized by PCR, using several oligonucleotides prepared to have overlapping portions with the ends of both CDR and FR as primers (see the method described in WO 98/13388). A humanized antibody is obtained by: ligating the resulting DNA to a DNA that encodes a human antibody constant region or a modified human antibody constant region; inserting the same into an expression vector; and introducing the vector into a host to produce the antibody

(see European Patent Application No. EP 239400 and International Patent Application Publication No. WO 96/02576).

[0075] Human antibody framework regions to be linked with CDR are selected so that the CDR forms a favorable antigen-binding site. If needed, amino acid substitution, deletion, addition and/or insertion may be introduced into the framework region of an antibody variable region.

[0076] A human antibody constant region, or an altered human antibody constant region in which one or more amino acids have been substituted, deleted, added, and/or inserted in a human antibody constant region, can be used as the constant region of a humanized antibody.

[0077] For example, C gamma 1, C gamma 2, C gamma 3, C gamma 4, C mu, C delta, C alpha 1, C alpha 2, and C epsilon can be used for the H chain, and C kappa and C lambda can be used for the L chain. The amino acid sequence of C kappa is shown in SEQ ID NO: 38, and the nucleotide sequence encoding this amino acid sequence is shown in SEQ ID NO: 37. The amino acid sequence of C gamma 1 is shown in SEQ ID NO: 40, and the nucleotide sequence encoding this amino acid sequence is shown in SEQ ID NO: 39. The amino acid sequence of C gamma 2 is shown in SEQ ID NO: 42, and the nucleotide sequence encoding this amino acid sequence is shown in SEQ ID NO: 41. The amino acid sequence of C gamma 4 is shown in SEQ ID NO: 44, and the nucleotide sequence encoding this amino acid sequence is shown in SEQ ID NO: 43.

[0078] Furthermore, human antibody C regions may be modified to improve antibody stability or antibody production stability. Human antibodies of any isotype such as IgG, IgM, IgA, IgE, or IgD may be used in antibody humanization; however, IgG is preferably used in the present invention. IgG1, IgG2, IgG3, IgG4, or the like can be used as the IgG.

[0079] Amino acids in the variable region (for example, CDR and FR) and constant region of a humanized antibody may be deleted, added, inserted, and/or substituted with amino acids after preparation. The antibodies in present invention also include such humanized antibodies comprising amino acid substitutions and the like.

[0080] The antibodies in present invention include not only divalent antibodies as represented by IgG, but also monovalent antibodies and multivalent antibodies as represented by IgM, as long as they have IL-6 receptor-binding activity and/or neutralizing activity. The multivalent antibodies of the present invention include multivalent antibodies in which the antigen-binding sites are all identical, and multivalent antibodies in which all or some of the antigen-binding sites are different. The antibodies in present invention include not only whole antibody molecules, but also minibodies and modified products thereof, as long as they bind to the IL-6 receptor

protein.

[0081] Minibodies are antibodies comprising an antibody fragment lacking a portion of a whole antibody (for example, whole IgG or such), and are not particularly limited as long as they have IL-6 receptor-binding activity and/or neutralizing activity and comprise an antibody fragment that lacks a portion of a whole antibody (for example, whole IgG or such). The minibodies in present invention are not particularly limited, as long as they comprise a portion of a whole antibody. However, the minibodies preferably comprise VH or VL, and particularly preferably comprise both VH and VL. Other preferable minibodies in present invention include, for example, minibodies comprising antibody CDRs. The minibodies may comprise all or some of the six CDRs of an antibody.

[0082] The minibodies in present invention preferably have a smaller molecular weight than whole antibodies. However, the minibodies may form multimers, for example, dimers, trimers, or tetramers, and thus their molecular weight is sometimes greater than that of whole antibodies.

[0083] Specifically, antibody fragments include, for example, Fab, Fab', F(ab')2, and Fv. Meanwhile, minibodies include, for example, Fab, Fab', F(ab')2, Fv, scFv (single chain Fv), diabodies, and sc(Fv)2 (single chain (Fv)2). Multimers (for example, dimers, trimers, tetramers, and polymers) of these antibodies are also included in the minibodies in present invention.

[0084] Antibody fragments can be obtained, for example, by treating antibodies with enzymes to produce antibody fragments. Enzymes known to generate antibody fragments include, for example, papain, pepsin, and plasmin. Alternatively, a gene encoding such antibody fragment can be constructed, introduced into an expression vector, and expressed in appropriate host cells (see, for example, Co, M.S. et al., J. Immunol. (1994) 152, 2968-2976; Better, M. & Horwitz, A. H. Methods in Enzymology (1989) 178, 476-496; Pluckthun, A. & Skerra, A. Methods in Enzymology (1989) 178, 476-496; Lamoyi, E., Methods in Enzymology (1989) 121, 652-663; Rousseaux, J. et al., Methods in Enzymology (1989) 121, 663-669; Bird, R. E. et al., TIBTECH (1991) 9, 132-137).

[0085] Digestive enzymes cleave at specific sites of an antibody fragment, yielding antibody fragments of specific structures shown below. Genetic engineering techniques can be applied to such enzymatically-obtained antibody fragments to delete an arbitrary portion of the antibody.

[0086] Antibody fragments obtained by using the above digestive enzymes are as follows.

Papain digestion: F(ab)2 or Fab

Pepsin digestion: F(ab')2 or Fab'

Plasmin digestion: Facb

[0087] The minibodies in present invention include antibody fragments lacking an arbitrary region, as long as they have IL-6 receptor-binding activity and/or neutralizing activity.

[0088] "Diabody" refers to a bivalent antibody fragment constructed by gene fusion (Holliger P et al., 1993, Proc. Natl. Acad. Sci. USA 90: 6444-6448; EP 404,097; WO 93/11161, etc). Diabodies are dimers composed of two polypeptide chains. In each of the polypeptide chains forming a dimer, a VL and a VH are generally linked by a linker in the same chain. In general, a linker in a diabody is short enough so that the VL and VH cannot bind to each other. Specifically, the number of amino acid residues constituting the linker is, for example, about five residues. Thus, the VL and VH encoded on the same polypeptide cannot form a single-chain variable region fragment, and will form a dimer with another single-chain variable region fragment. As a result, the diabody has two antigen binding sites.

[0089] ScFv antibodies are single-chain polypeptides produced by linking VH and VL via a linker or such (Huston, J. S. et al., Proc. Natl. Acad. Sci. U.S.A. (1988) 85, 5879-5883; Plueckthun "The Pharmacology of Monoclonal Antibodies" Vol. 113, eds., Resenburg and Moore, Springer Verlag, New York, pp. 269-315, (1994)). The H-chain V region and L-chain V region of scFv may be derived from any antibody described herein. The peptide linker for linking the V regions is not particularly limited. For example, an arbitrary single-chain peptide containing about three to 25 residues can be used as the linker. Specifically, it is possible to use the peptide linkers described below or such.

[0090] The V regions of the two chains can be linked, for example, by PCR as described above. First, a DNA encoding the complete amino acid sequence or a desired partial amino acid sequence of one of the DNAs shown below is used as a template to link the V regions by PCR:

a DNA sequence encoding an H chain or H-chain V region of an antibody, and

a DNA sequence encoding an L chain or L-chain V region of an antibody.

[0091] DNAs encoding the V region of an H chain or L chain are amplified by PCR using a pair of primers containing corresponding sequences of the two ends of the DNA to be amplified. Then, a DNA encoding the peptide linker portion is prepared. The peptide linker-encoding DNA can also be synthesized by PCR. A nucleotide sequence that can be used to link the separately synthesized amplification products of V region is added to the 5' end of the primers to be used. Then, PCR is carried out using each of the DNAs in [H chain V region DNA] - [peptide linker DNA] - [L chain V region DNA] and assembly PCR primers.

[0092] The assembly PCR primers contain a combination of a primer that anneals with the 5' end of the [H chain V region DNA] and a primer that anneals with the 3' end of the [L chain V region DNA]. In other words, the assembly PCR primers are a set of primers that can be used to amplify DNAs encoding the full-length sequence of the scFv to be

synthesized. Meanwhile, nucleic sequences that can be used to link each of the V-region DNAs are added to the [peptide linker DNA]. Then, these DNAs are linked, and then the whole scFv is ultimately generated as an amplification product using the assembly PCR primers. Once the scFv-encoding DNAs are generated, expression vectors containing these DNAs and recombinant cells transformed with these expression vectors can be obtained by conventional methods. Further, the scFv can be obtained through expression of the scFv-encoding DNAs by culturing the resulting recombinant cells.

[0093] The order of VH and VL to be linked is not particularly limited, and they may be arranged in any order. Examples of the arrangement are listed below.

[VH] linker [VL]

[VL] linker [VH]

[0094] sc(Fv)2 is a single-chain minibody produced by linking two VHs and two VLs using linkers and such (Hudson et al., 1999, J Immunol. Methods 231:177-189). Sc(Fv)2 can be produced, for example, by linking scFv using a linker.

[0095] Preferably, the two VHs and two VLs of an antibody are arranged in the order of VH, VL, VH, and VL ([VH] linker [VL] linker [VH] linker [VL]) from the N terminus of the single-chain polypeptide; however, the order of the two VHs and two VLs is not limited to the above arrangement, and they may be arranged in any order. Examples of the arrangement are listed below:

[VL] linker [VH] linker [VH] linker [VL]

[VH] linker [VL] linker [VL] linker [VH]

[VH] linker [VH] linker [VL] linker [VL]

[VL] linker [VL] linker [VH] linker [VH]

[VL] linker [VH] linker [VL] linker [VH]

[0096] The amino acid sequence of the minibody VH or VL may contain substitutions, deletions, additions, and/or insertions. Furthermore, as long as VH and VL have antigen-binding activity when assembled, a portion may be deleted or other polypeptides may be added. Moreover, the variable regions may be chimerized or humanized.

[0097] In present invention, linkers that can be used to link the antibody variable regions include arbitrary peptide linkers that can be introduced by genetic engineering, and synthetic linkers, for example, the linkers disclosed in Protein Engineering, (1996) 9(3), 299-305.

[0098] The preferred linkers in present invention are peptide linkers. The length of the peptide linkers is not particularly limited and those skilled in the art can appropriately select the length according to the purpose. The typical length is one to 100 amino acids, preferably 3 to 50 amino acids, more preferably 5 to 30 amino acids, and par-

ticularly preferably 12 to 18 amino acids (for example, 15 amino acids).

[0099] For example, amino acid sequences for peptide linkers include the following sequences:

Ser
Gly Ser
Gly Gly Ser
Ser Gly Gly
Gly Gly Gly Ser (SEQ ID NO: 45)
Ser Gly Gly Gly (SEQ ID NO: 46)
Gly Gly Gly Gly Ser (SEQ ID NO: 47)
Ser Gly Gly Gly Gly (SEQ ID NO: 48)
Gly Gly Gly Gly Gly Ser (SEQ ID NO: 49)
Ser Gly Gly Gly Gly Gly (SEQ ID NO: 50)
Gly Gly Gly Gly Gly Gly Ser (SEQ ID NO: 51)
Ser Gly Gly Gly Gly Gly Gly (SEQ ID NO: 52)
(Gly Gly Gly Gly Ser [SEQ ID NO: 47])n
(Ser Gly Gly Gly Gly [SEQ ID NO: 48])n
where n is an integer of 1 or more.

The amino acid sequences of peptide linkers can be appropriately selected by those skilled in the art according to the purpose. For example, the above "n" which determines the length of the peptide linker is typically one to five, preferably one to three, and more preferably one or two.

[0100] Synthetic linkers (chemical crosslinking agents) include, crosslinking agents routinely used to crosslink peptides, for example, N-hydroxysuccinimide (NHS), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl)suberate (BS3), dithiobis(succinimidyl propionate) (DSP), dithiobis(sulfosuccinimidyl propionate) (DTSSP), ethylene glycol bis(succinimidyl succinate) (EGS), ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS), disuccinimidyl tartarate (DST), disulfosuccinimidyl tartarate (sulfo-DST), bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone (BSOCOES), and bis[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone (sulfo-BSOCOES). These crosslinking agents are commercially available.

[0101] In general, three linkers are required to link four antibody variable regions. These multiple linkers may be the same or different linkers.

[0102] The antibodies in present invention also include antibodies in which one or more amino acid residues have been added to the amino acid sequence of an antibody. Furthermore, the antibodies in present invention also include fusion proteins in which an above-described antibody is fused with another peptide or protein. The fusion protein can be prepared by ligating a polynucleotide encoding an antibody and a polynu-

cleotide encoding another peptide or polypeptide in frame, introducing this DNA into an expression vector, and expressing this vector in a host. Techniques known to those skilled in the art can be used. The peptide or polypeptide to be fused with an antibody of the present invention may be a known peptide, for example, FLAG (Hopp, T. P. et al., BioTechnology 6, 1204-1210 (1988)), 6x His consisting of six His (histidine) residues, 10x His, influenza hemagglutinin (HA), human c-myc fragment, VSV-GP fragment, p18HIV fragment, T7-tag, HSV-tag, E-tag, SV40 T antigen fragment, lck tag, alpha-tubulin fragment, B-tag, and Protein C fragment. Polypeptides to be fused with the antibodies of the present invention include, for example, GST (glutathione-S-transferase), HA (influenza hemagglutinin), immunoglobulin constant region, beta-galactosidase, and MBP (maltose-binding protein). Commercially available polynucleotides encoding these peptides or polypeptides can be fused with a polynucleotide encoding an antibody as described in present invention. A fusion polypeptide can be prepared by expressing the so obtained fusion polynucleotide.

[0103] Moreover, the antibodies described in present invention may also be conjugated antibodies linked to various molecules such as polymers, including polyethylene glycol (PEG) and hyaluronic acid; radioactive substances; fluorescent substances; luminescent substances; enzymes; and toxins. Such conjugated antibodies can be obtained by chemically modifying the obtained antibodies. Methods for antibody modification are already established in the art (see, for example, US 5,057,313 and US 5,156,840). The "antibodies" in present invention also include such conjugated antibodies.

[0104] Furthermore, the antibodies in present invention include antibodies with altered sugar chains.

[0105] Furthermore, the antibodies used in present invention may be bispecific antibodies. Bispecific antibody refers to an antibody that has variable regions that recognize different epitopes in the same antibody molecule. A bispecific antibody in present invention may be a bispecific antibody that recognizes different epitopes on the IL-6 receptor molecule, or a bispecific antibody in which one of the antigen-binding sites recognizes the IL-6 receptor and the other antigen-binding site recognizes another substance. Examples of antigens that bind to the other antigen-binding site of a bispecific antibody that comprises an IL-6 receptor-recognizing antibody of the present invention include IL-6, TNF alpha, TNFR1, TNFR2, CD80, CD86, CD28, CD20, CD19, IL-1 alpha, IL-beta, IL-1R, RANKL, RANK, IL-17, IL-17R, IL-23, IL-23R, IL-15, IL-15R, BlyS, lymphotoxin alpha, lymphotoxin beta, LIGHT ligand, LIGHT, VLA-4, CD25, IL-12, IL-12R, CD40, CD40L, BAFF, CD52, CD22, IL-32, IL-21, IL-21R, GM-CSF, GM-CSFR, M-CSF, M-CSFR, IFN-alpha, VEGF, VEGFR, EGF, EGFR, CCR5, APRIL, and APRILR.

[0106] Methods for producing bispecific antibodies are known. Bispecific antibodies can be prepared, for example, by linking two types of antibodies recognizing different antigens. Antibodies to be linked may be a half molecule each containing an H chain and an L chain, or a quarter molecule containing only one H chain. Alternatively, fusion cells producing bispecific antibodies can be prepared by fusing hybridomas producing different monoclonal antibodies. Furthermore, bispecific antibodies can be produced by genetic engineering techniques.

[0107] As described below, the antibodies used in present invention may differ in amino acid sequence, molecular weight, isoelectric point, presence/absence of sugar chains, and conformation, depending on the purification method, or the cell or host used to produce the antibodies. However, as long as the antibody obtained is functionally equivalent to an antibody described in present invention, it should be considered as included in present invention. For example, when an antibody is expressed in prokaryotic cells, for example, Escherichia coli, a methionine residue is added to the N terminus of the original antibody amino acid sequence. Such antibodies are also included in the antibodies described in present invention.

[0108] Polypeptides of anti-IL-6 receptor antibodies and such in present invention can be produced by methods known to those skilled in the art.

[0109] An anti-IL-6 receptor antibody can be prepared, for example, by genetic recombination techniques known to those skilled in the art based on the sequence of the anti-IL-6 receptor antibody obtained. Specifically, an anti-IL-6 receptor antibody can be prepared by constructing a polynucleotide encoding the antibody based on the sequence of an IL-6 receptor-recognizing antibody, inserting the polynucleotide into an expression vector, and then expressing it in an appropriate host cell (see for example, Co, M. S. et al., J. Immunol. (1994) 152, 2968-2976; Better, M. and Horwitz, A. H., Methods Enzymol. (1989) 178, 476-496; Pluckthun, A. and Skerra, A., Methods Enzymol. (1989) 178, 497-515; Lamoyi, E., Methods Enzymol. (1986) 121, 652-663; Rousseaux, J. et al., Methods Enzymol. (1986) 121, 663-669; Bird, R. E. and Walker, B. W., Trends Biotechnol. (1991) 9, 132-137).

[0110] Thus, the present invention provides methods of producing (i) a polypeptide of the present invention, or (ii) a polypeptide encoded by a gene encoding the polypeptide of the present invention, wherein the methods comprise the step of culturing a host cell comprising a vector into which a polynucleotide encoding the polypeptide of the present invention is introduced.

[0111] More specifically, the present invention provides methods of producing a polypeptide of the present invention, which comprise the steps of:

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

(b) obtaining the polypeptide encoded by the gene.

[0112] Examples of the vector include M13-type vectors, pUC-type vectors, pBR322, pBluescript, and pCR-Script. Alternatively, when the objective is to subclone and excise the cDNA, other examples of the vector in addition to the ones described above include pGEM-T, pDIRECT, and pT7. Expression vectors are particularly useful for producing antibodies described in present invention. For example, when the expression vector is used for expression in E. coli, the vector should have features that allow its amplification in E. coli. In addition, when the host is E. coli such as JM109, DH5 alpha, HB101, or XL1-Blue, it is essential that the vector carries a promoter that allows its efficient expression in E. coli, for example, lacZ promoter (Ward et al., Nature (1989) 341, 544-546; FASEB J. (1992) 6, 2422-2427), araB promoter (Better et al., Science (1988) 240, 1041-1043), T7 promoter or such. Such vector includes pGEX-5X-1 (Pharmacia), "QIAexpress system" (Quiagen), pEGFP, and pET (in this case, the host is preferably BL21 which expresses T7 RNA polymerase), in addition to the ones described above.

[0113] Furthermore, the expression plasmid vectors may contain signal sequences for antibody secretion. As a signal sequence for antibody secretion, the pelB signal sequence (Lei, S. P. et al., J. Bacteriol. (1987) 169, 4379) may be used for production into the E. coli periplasm. The vectors can be introduced into host cells, for example, by calcium chloride methods or electroporation.

[0114] In addition to vectors for E. coli, the vectors for producing antibodies described in present invention include, for example, mammal-derived expression vectors (for example, pcDNA3 (Invitrogen), pEF-BOS (Nucleic Acids. Res. (1990) 18(17), p5322), pEF, and pCDM8), insect cell-derived expression vectors (for example, the "Bac-to-BAC baculovirus expression system" (Gibco-BRL) and pBacPAK8), plant-derived expression vectors (for example, pMH1 and pMH2), animal virus-derived expression vectors (for example, pHSV, pMV, and pAdexLcw), retrovirus-derived expression vectors (for example, pZIPneo), yeast-derived expression vectors (for example, "Pichia Expression Kit" (Invitrogen), pNV11, and SP-Q01), and Bacillus subtilis-derived expression vectors (for example, pPL608 and pKTH50).

[0115] When the expression plasmid vector is used for expression in animal cells such as CHO, COS, and NIH3T3 cells, it must have a promoter necessary for expression in those cells, for example, SV40 promoter (Mulligan et al., Nature (1979) 277, 108), MMLV-LTR promoter, EF1 alpha promoter (Mizushima et al., Nucleic Acids Res. (1990) 18, 5322), or CMV promoter. It is even more preferable if the vector has a gene for selection of transformed cells (for example, a drug resistance gene that allows distinction by an agent (neomycin, G418, or such). Vectors with such characteristics include, for example, pMAM, pDR2, pBK-RSV, pBK-CMV, pOPRSV, and pOP13.

[0116] In addition, when the objective is to stably express genes and amplify a gene's copy number in the cells, a method in which CHO cells deficient in a nucleic acid synthesis pathway are introduced with a vector having a DHFR gene which compensates for the deficiency (for example, pSV2-dhfr ("Molecular Cloning 2nd edition" Cold Spring Harbor Laboratory Press, (1989))) and the vector is amplified using methotrexate (MTX) can be used. Further, when the objective is transient gene expression, a method in which COS cells carrying a gene expressing the SV40 T antigen on their chromosome are transformed with a vector carrying an SV40 replication origin (pcD and such) can be used. It is possible to use replication origins derived from polyoma virus, adenovirus, bovine papilloma virus (BPV), and such. Moreover, to amplify the gene copy number in host cell lines, the expression vectors may comprise the aminoglycoside transferase (APH) gene, thymidine kinase (TK) gene, E. coli xanthine-guanine phosphoribosyltransferase (Ecogpt) gene, dihydrofolate reductase (dhfr) gene, and such as a selection marker.

[0117] The resulting antibodies described in present invention can be isolated from host cells or from outside the cells (the medium, or such), and purified as substantially pure and homogenous antibodies. The antibodies can be separated and purified using conventional separation and purification methods for antibody purification, without being limited thereto. For example, the antibodies can be separated and purified by appropriately selecting and combining column chromatography, filtration, ultrafiltration, salting out, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectrofocusing, dialysis, recrystallization, and such.

[0118] Chromatography includes, for example, affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel filtration, reverse phase chromatography, and adsorption chromatography (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak et al., Cold Spring Harbor Laboratory Press, 1996). These chromatographies can be carried out using liquid-phase chromatography, for example, HPLC and FPLC. Columns used for affinity chromatography include protein A columns and protein G columns. Examples of columns using Protein A include Hyper D, POROS, and Sepharose FF (GE Amersham Biosciences). The present invention also includes antibodies highly purified using such purification methods.

[0119] The IL-6 receptor binding activity of the obtained antibodies can be measured by methods known to those skilled in the art. Methods for measuring the antigen-binding activity of an antibody include, for example, enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), radioimmunoassay (RIA), and fluorescent antibody methods. For example, when enzyme immunoassay is used, antibody-

containing samples such as purified antibodies and culture supernatants of antibody-producing cells are added to antigen-coated plates. A secondary antibody labeled with an enzyme such as alkaline phosphatase is added, and the plates are incubated. After washing, an enzyme substrate such as p-nitrophenyl phosphate is added, and the absorbance is measured to evaluate the antigen-binding activity.

[0120] Pharmaceutical compositions

The present invention provides pharmaceutical compositions that comprise an above-described polypeptide as an active ingredient. The pharmaceutical compositions of present invention can be used for IL-6-associated diseases such as rheumatoid arthritis. Thus, the present invention also provides agents for treating diseases such as rheumatoid arthritis, which comprise an antibody described above as an active ingredient. Preferred examples of target diseases include, but are not limited to, rheumatoid arthritis, juvenile idiopathic arthritis, systemic juvenile idiopathic arthritis, Castleman's disease, systemic lupus erythematosus (SLE), lupus nephritis, Crohn's disease, lymphoma, ulcerative colitis, anemia, vasculitis, Kawasaki disease, Still's disease, amyloidosis, multiple sclerosis, transplantation, age-related macular degeneration, ankylosing spondylitis, psoriasis, psoriatic arthritis, chronic obstructive pulmonary disease (COPD), IgA nephropathy, osteoarthritis, asthma, diabetic nephropathy, GVHD, endometriosis, hepatitis (NASH), myocardial infarction, arteriosclerosis, sepsis, osteoporosis, diabetes, multiple myeloma, prostate cancer, kidney cancer, B-cell non-Hodgkin's lymphoma, pancreatic cancer, lung cancer, esophageal cancer, colon cancer, cancer cachexia, cancer neuroinvasion, myocardial infarction, myopic choroidal neovascularization, idiopathic choroidal neovascularization, uveitis, chronic thyroiditis, delayed hypersensitivity, contact dermatitis, atopic dermatitis, mesothelioma, polymyositis, dermatomyositis, panuveitis, anterior uveitis, intermediate uveitis, scleritis, keratitis, orbital inflammation, optic neuritis, diabetic retinopathy, proliferative vitreoretinopathy, dry eye, and post-operative inflammation.

[0121] The phrase "to comprise an anti-IL-6 receptor antibody as an active ingredient" means comprising an anti-IL-6 receptor antibody as at least one of the active ingredients, without particular limitation on its content. Furthermore, the pharmaceutical compositions of present invention may contain other active ingredients in combination with the polypeptides described above.

[0122] The pharmaceutical compositions of present invention may be used not only for therapeutic purposes, but also for preventive purposes.

[0123] Amino acids contained in the amino acid sequences in the present invention may be post-translationally modified. For example, the modification of an N-terminal glutamine (Gln) residue into a pyroglutamic acid (pGlu) residue by pyroglutamylation is well-known to those skilled in the art. Naturally, such post-translationally modified

amino acids are included in the amino acid sequences in the present invention.

[0124] Sugar chains that are bound to the antibodies according to the present invention may be of any structure. A sugar chain at position 297 (EU numbering) may be of any sugar chain structure (preferably a fucosylated sugar chain), or no sugar chain may exist at that position (for example, this can be achieved by producing antibodies in Escherichia coli or by introducing alteration so that no sugar chain binds to position 297, EU numbering).

[0125] All prior art references cited herein are incorporated by reference into this description.

Examples

[0126] Herein below, the present invention will be described with the aid of examples, but shall not be understood as being limited thereto.

Example 1

[0127] Identification of mutation sites in the variable regions for enhancing the affinity of TOCILIZUMAB for IL-6 receptor

A library of CDR sequences into which mutations have been introduced was constructed and assayed to improve the affinity of TOCILIZUMAB (H chain WT-IgG1/SEQ ID NO: 53; L chain WT-kappa/SEQ ID NO: 54) for IL-6 receptor. Screening of a library of CDR mutations revealed mutations that improve the affinity for IL-6 receptor. The mutations are shown in Fig. 1. A combination of these mutations yielded high-affinity TOCILIZUMAB such as RDC-23 (H chain RDC23H-IgG1/SEQ ID NO: 55; L chain RDC-23L-kappa/SEQ ID NO: 56). The affinity for soluble IL-6 receptor and biological activity determined using BaF/gp130 were compared between RDC-23 and TOCILIZUMAB (see Reference Examples for the method).

[0128] The result of affinity measurement is shown in Table 1. The result of biological activity determination using BaF/gp130 (the final concentration of IL-6 was 30 ng/ml) is shown in Fig. 2. The results showed that the affinity of RDC-23 was about 60 times higher, and the activity expressed as concentration for 100% inhibition of BaF/gp130 was about 100 times higher when compared to TOCILIZUMAB.

[0129] [Table 1]

	k_a(1/Ms)	k_d(1/s)	KD (M)
TOCILIZUMAB	4.9E+05	2.0E-03	4.0E-09
RDC-23	8.4E+05	4.3E-05	6.7E-11

Example 2

[0130] Identification of mutations for improving the pharmacokinetics of TOCILIZUMAB via reduction of its isoelectric point

To improve the pharmacokinetics of TOCILIZUMAB, investigation was carried out

to identify mutation sites that would decrease the isoelectric point of the variable regions without significantly reducing the binding to the IL-6 receptor. Screening of mutation sites in the variable regions, which were predicted based on a three-dimensional structure model of TOCILIZUMAB, revealed mutation sites that would decrease the isoelectric point of the variable regions without significantly reducing its binding to the IL-6 receptor. These are shown in Fig. 3. A combination of these mutations yielded TOCILIZUMAB with reduced isoelectric point including, for example, H53/L28 (H chain H53-IgG1/SEQ ID NO: 57; L chain L28-kappa/SEQ ID NO: 58). The affinity for soluble IL-6 receptor, isoelectric point, pharmacokinetics in mice, and biological activity determined using BaF/gp130 were compared between H53/L28 and TOCILIZUMAB (see Reference Examples for the method).

[0131] The result of affinity measurement is shown in Table 2. The measurement result for the biological activity obtained using BaF/gp130 (the final concentration of IL-6 was 30 ng/ml) is shown in Fig. 4. The results showed that the affinity of H53/L28 was about six times higher and the activity expressed as concentration for 100% inhibition of BaF/gp130 was about several times higher when compared to TOCILIZUMAB.

[0132] [Table 2]

	k_a(1/Ms)	k_d(1/s)	KD (M)
TOCILIZUMAB	4.9E+05	2.0E-03	4.0E-09
H53/L28	7.6E+05	5.2E-04	6.8E-10

[0133] The result of isoelectric point determination by isoelectric point electrophoresis known to those skilled in the art showed that the isoelectric points of TOCILIZUMAB and H53/L28 were about 9.3 and 6.5 to 6.7, respectively. Thus, the isoelectric point of H53/L28 was reduced by about 2.7 when compared to TOCILIZUMAB. Furthermore, the theoretical isoelectric point of the VH/VL variable regions was calculated using GENETYX (GENETYX CORPORATION). The result showed that the theoretical isoelectric points of TOCILIZUMAB and H53/L28 were 9.20 and 4.52, respectively. Thus, the isoelectric point of H53/L28 was reduced by about 4.7 when compared to TOCILIZUMAB.

[0134] To assess the pharmacokinetics of the altered antibody H53/L28 which has a reduced isoelectric point, the pharmacokinetics of TOCILIZUMAB and H53/L28 in normal mice were compared. A single dose of TOCILIZUMAB or H53/L28 was intravenously (IV) or subcutaneously (SC) administered at 1 mg/kg to mice (C57BL/6J; Charles River Japan, Inc.) to evaluate the time course of plasma concentration. The time courses of plasma concentration for TOCILIZUMAB and H53/L28 after intravenous administration or subcutaneous administration are shown in Figs. 5 and 6, respectively. Pharmacokinetic parameters (clearance (CL) and half-life (T1/2)) obtained using WinNonlin (Pharsight) are shown in Table 3. The plasma half-life (T1/2) of H53/L28

after intravenous administration was prolonged to about 1.3 times that of TOCILIZUMAB, while the clearance was reduced by about 1.7 times. T1/2 of H53/L28 after subcutaneous administration was increased to about twice that of TOCILIZUMAB, while the clearance was reduced by about 2.1 times. Thus, it was found that the pharmacokinetics could be significantly improved by reducing the iso-electric point of TOCILIZUMAB through amino acid substitution.

[0135] [Table 3]

	IV		SC	
	CL mL/h/kg	T1/2 day	CL/F mL/h/kg	T1/2 day
TOCILIZUMAB	0.177	18.5	0.18	14.7
H53/L28	0.102	23.5	0.086	29.7

Example 3

[0136] Identification of mutation sites that reduce the immunogenicity of TOCILIZUMAB

Identification of mutations that reduce the immunogenicity risk of T-cell epitopes present in the variable regions

T-cell epitopes present in the variable-region sequence of TOCILIZUMAB were analyzed using TEPITOPE (Methods. 2004 Dec; 34(4):468-75). As a result, the L-chain CDR2 was predicted to have many T-cell epitopes that would bind to HLA (i.e. to have a sequence with a high immunogenicity risk). Thus, TEPITOPE analysis was carried out to examine amino acid substitutions that would reduce the immunogenicity risk of the L-chain CDR2 without decreasing the stability, binding activity, or neutralizing activity.

[0137] As described below, the screening result demonstrated that the immunogenicity risk can be reduced without decreasing the stability, binding activity, or neutralizing activity by substituting the threonine at L51 (Kabat's numbering; Kabat EA et al., (1991) Sequences of Proteins of Immunological Interest, NIH)) of the L chain CDR2 (SEQ ID NO: 59) of TOCILIZUMAB with glycine, and the arginine at L53 with glutamic acid (SEQ ID NO: 60).

TOCILIZUMAB L-chain CDR2 (SEQ ID NO: 59)

TOCILIZUMAB L-chain CDR2 with T-cell epitopes removed (SEQ ID NO: 60)

Example 4

[0138] Reduction of immunogenicity risk by full humanization of the variable region framework sequences of TOCILIZUMAB

In the process of TOCILIZUMAB humanization, some mouse sequences remain in the framework sequence to maintain binding activity (Cancer Res. 1993 Feb 15; 53(4):851-6). These sequences are H27, H28, H29, and H30 in the H-chain FR1, and

H71 in the H-chain FR3 (Kabat's numbering; Kabat EA et al., (1991) Sequences of Proteins of Immunological Interest, NIH)) of the variable region sequence of TOCILIZUMAB. The mouse sequences that remained are a potential cause of increased immunogenicity risk. Thus, it was assessed whether the framework sequence could be fully humanized to further reduce the immunogenicity risk of TOCILIZUMAB.

[0139] The result showed that the entire framework of TOCILIZUMAB could be completely humanized without decreasing the stability, binding activity, or neutralizing activity, by substituting the H-chain FR1 (SEQ ID NO: 61) of TOCILIZUMAB with the humanized H-chain FR1-A (SEQ ID NO: 62) shown below, and substituting the-H chain FR3 (SEQ ID NO: 63) with the humanized H chain FR3 (SEQ ID NO: 64) shown below.

TOCILIZUMAB H chain FR1 (SEQ ID NO: 61)

Humanized H chain FR1-A (SEQ ID NO: 62) (derived from germline IMGT hVH_4)

TOCILIZUMAB H chain FR3 (SEQ ID NO: 63)

Humanized H chain FR3 (SEQ ID NO: 64) (derived from Mol. Immunol. 2007, 44(4):412-422)

Example 5

[0140] Identification of mutation sites to improve the pharmacokinetics based on pH-dependent binding of TOCILIZUMAB to the IL-6 receptor

One of the methods for improving the pharmacokinetics of TOCILIZUMAB is to improve the molecule such that a single molecule of TOCILIZUMAB would repeatedly bind and neutralize several molecules of the IL-6 receptor. It is assumed that after binding to membrane-type IL-6 receptor, TOCILIZUMAB is taken up into intracellular endosomes via internalization while bound to membrane-type IL-6 receptor, then transferred into lysosomes while bound to membrane-type IL-6 receptor, and becomes degraded by lysosomes. Specifically, one molecule of TOCILIZUMAB typically binds to one or two molecules of membrane-type IL-6 receptor (in a monovalent or divalent manner) and is degraded in lysosomes after internalization. Therefore, one molecule of TOCILIZUMAB can only bind and neutralize one or two molecules of membrane-type IL-6 receptor.

[0141] Thus, the present inventors thought that if it were possible to create TOCILIZUMAB that binds in a pH-dependent manner, in which the binding of TOCILIZUMAB is maintained under neutral conditions but significantly reduced under acidic conditions, TOCILIZUMAB which binds in a pH-dependent manner could dissociate from membrane-type IL-6 receptor (antigen) in the endosomes and return to the plasma by binding to FcRn present in the endosomes, as illustrated in Fig. 7. Once returned to the

plasma, TOCILIZUMAB which binds in a pH-dependent manner could again bind to membrane-type IL-6 receptor. By repeating this binding in the plasma and dissociation in the endosomes, it is thought that one molecule of TOCILIZUMAB can repeatedly bind/neutralize several molecules of the IL-6 receptor. Thus, TOCILIZUMAB which binds in a pH-dependent manner is assumed to have improved pharmacokinetics as compared to TOCILIZUMAB.

[0142] For TOCILIZUMAB to dissociate from the IL-6 receptor under the acidic condition in the endosome, the binding must be significantly weakened under the acidic condition as compared to under the neutral condition. On the cell surface, strong IL-6 receptor binding is required for neutralization; therefore, at pH 7.4 which is the cell surface pH, the antibody must bind to the IL-6 receptor as strongly as or more strongly than TOCILIZUMAB. It has been reported that the endosomal pH is generally 5.5 to 6.0 (Nat Rev Mol Cell Biol. 2004 Feb;5(2):121-32). Thus, if TOCILIZUMAB which binds in a pH-dependent manner is modified to weakly bind to the IL-6 receptor at pH 5.5 to 6.0, it can be predicted to dissociate from the IL-6 receptor under the acidic condition in the endosomes. Specifically, if TOCILIZUMAB which binds in a pH-dependent manner is improved to strongly bind to the IL-6 receptor at pH 7.4, which is the cell surface pH, and to weakly bind to IL-6 receptor at pH 5.5 to 6.0, which is the endosomal pH, one molecule of TOCILIZUMAB can bind and neutralize several molecules of the IL-6 receptor, and the pharmacokinetics can therefore be improved.

[0143] A possible method for conferring pH dependence on the binding of TOCILIZUMAB to the IL-6 receptor is to introduce histidine residues into the variable region of TOCILIZUMAB, since the pKa of a histidine residue is about 6.0 to 6.5, and its state of proton dissociation changes between neutral (pH 7.4) and acidic (pH 5.5 to 6.0) conditions. Thus, screening was carried out to identify sites for histidine introduction in the variable regions based on a three-dimensional structure model of TOCILIZUMAB. Furthermore, selected variable region sequences of TOCILIZUMAB were randomly substituted with histidine to design a library for screening. The screening was carried out using the binding to the IL-6 receptor at pH 7.4 and dissociation from the IL-6 receptor, or the reduction of affinity at pH 5.5 to 5.8 as an index.

[0144] As a result, the present inventors discovered mutation sites that confer the binding of TOCILIZUMAB to the IL-6 receptor with pH dependency (the property to bind at pH 7.4 and dissociate at pH 5.8). These are shown in Fig. 8. In Fig. 8, the substitution of tyrosine at H27 to histidine is a mutation in the H-chain FR1, not in the CDR. However, as described in Eur. J. Immunol. (1992) 22: 1719-1728, a sequence with histidine at H27 is a human sequence (SEQ ID NO: 65). Thus, the antibody can be completely humanized by using the following framework in combination with

Example 4.

[0145] Humanized H-chain FR1-B (SEQ ID NO: 65)

A combination of mutations including, for example, H3pI/L73 (H chain H3pI-IgG1/SEQ ID NO: 66; L chain L73-kappa/SEQ ID NO: 67) can yield TOCILIZUMAB with pH-dependent binding properties. H3pI/L73 and TOCILIZUMAB were compared for their affinity towards soluble IL-6 receptor at pH 7.4, rate of dissociation from membrane-type IL-6 receptor at pH 7.4 and pH 5.8, biological activity using BaF/gp130, and pharmacokinetics in cynomolgus monkey and human IL-6 receptor transgenic mice (see Reference Examples for the method).

[0146] The result of affinity assay for soluble IL-6 receptor at pH 7.4 is shown in Table 4. The assay result for the biological activity obtained using BaF/gp130 (final IL-6 concentration of 30 ng/ml) is shown in Fig. 9. These results showed that H3pI/L73 is comparable to TOCILIZUMAB in terms of affinity for soluble IL-6 receptor at pH 7.4 and activity on BaF/gp130.

[0147] [Table 4]

	k_a(1/Ms)	k_d(1/s)	KD (M)
TOCILIZUMAB	5.1E+05	1.0E-03	2.1E-09
H3pI/L73	5.4E+05	7.4E-04	1.4E-09

[0148] The measurement result for the rate of dissociation of TOCILIZUMAB or H3pI/L73 from membrane-type IL-6 receptor at pH 7.4 and pH 5.8 is shown in Table 5. As compared to TOCILIZUMAB, the dissociation rate of H3pI/L73 at pH 5.8 was faster and the pH dependence of the rate of dissociation from membrane-type IL-6 receptor was increased by about 2.6 times.

[0149] [Table 5]

	pH7.4	pH5.8	$k_{d(pH5.8)} / k_{d(pH7.4)}$
	k_d(1/s)	k_d(1/s)	pH DEPENDENCY
TOCILIZUMAB	2.5E-04	2.5E-04	1.00
H3pI/L73	2.6E-04	6.7E-04	2.59

[0150] A single dose of TOCILIZUMAB or H3pI/L73 was intravenously administered at 1 mg/kg to cynomolgus monkeys to assess the time course of plasma concentration. The plasma concentration time courses of TOCILIZUMAB or H3pI/L73 after intravenous administration are shown in Fig. 10. The result showed that the pharmacokinetics of H3pI/L73 in cynomolgus monkeys was significantly improved as compared to TOCILIZUMAB.

[0151] A single dose of TOCILIZUMAB or H3pI/L73 was intravenously administered at 25 mg/kg to human IL-6 receptor transgenic mice (hIL-6R tg mice; Proc Natl Acad Sci U S A. 1995 May 23; 92(11):4862-6) to assess the time course of plasma concentration.

The plasma concentration time courses of TOCILIZUMAB or H3pI/L73 after intravenous administration are shown in Fig. 11. The result showed that the pharmacokinetics of H3pI/L73 in human IL-6 receptor transgenic mice was significantly improved as compared to TOCILIZUMAB.

[0152] H3pI/L73, which is a TOCILIZUMAB with pH-dependent binding properties, showed significantly improved pharmacokinetics in cynomolgus monkeys and human IL-6 receptor transgenic mice when compared to TOCILIZUMAB. This suggests that it is possible to bind to and neutralize several molecules of the IL-6 receptor with one single molecule, by conferring the property of binding an antigen at pH 7.4 and dissociating from the antigen at pH 5.8. It was also considered that the pharmacokinetics could be further improved by conferring IL-6 receptor binding with a more pronounced pH dependence than that of H3pI/L73.

Example 6

[0153] Optimization of the TOCILIZUMAB constant region

Reduction of the heterogeneity of TOCILIZUMAB H-chain C terminus

For heterogeneity of the H-chain C-terminal sequences of an IgG antibody, deletion of the C-terminal amino acid lysine residue, and amidation of the C-terminal carboxyl group due to deletion of both of the two C-terminal amino acids, glycine and lysine, have been reported (Anal Biochem. 2007 Jan 1; 360(1):75-83). Also in TOCILIZUMAB, the major component is a sequence in which the C-terminal amino acid lysine in the nucleotide sequence is deleted by post-translational modification; however, sub-components in which the lysine remains and sub-components in which the C-terminal carboxyl group is amidated due to deletion of both glycine and lysine also contribute to heterogeneity. It is not easy and would be more costly to manufacture them as a pharmaceutical in large-scale while maintaining the objective substances/related substances related heterogeneity between productions. If possible, it is desirable to be single substances, and to have reduced heterogeneity when developing antibodies as pharmaceuticals. Thus, it is preferable that the H-chain C-terminal heterogeneity is absent when developing antibodies as pharmaceuticals.

[0154] The C-terminal amino acid was altered to reduce the C-terminal amino acid heterogeneity. The result showed that the C-terminus-derived heterogeneity can be prevented by pre-deleting from the nucleotide sequence, the lysine and glycine residues at the C terminus of the H-chain constant region of TOCILIZUMAB. TOCILIZUMAB, TOCILIZUMAB that lacks the C-terminal lysine residue (TOCILIZUMAB delta K: H chain WT-IgG1 delta K/SEQ ID NO: 68; L chain WT-kappa/SEQ ID NO: 54), and TOCILIZUMAB that lacks the C-terminal lysine and glycine residues (TOCILIZUMAB delta GK: H chain WT-IgG1 delta GK/SEQ ID

NO: 69; L chain WT-kappa/SEQ ID NO: 54) were assessed for heterogeneity by cation exchange chromatography. The ProPac WCX-10, 4x250 mm (Dionex) column was used; and mobile phase A was 25 mmol/L MES/NaOH (pH 6.1) and mobile phase B was 25 mmol/L MES/NaOH, 250 mmol/L NaCl (pH 6.1). Appropriate flow rate and gradient were used. The assessment result obtained by cation exchange chromatography is shown in Fig. 12. The result showed that the C-terminal amino acid heterogeneity can be reduced by pre-deleting from the nucleotide sequence, both the lysine and glycine residues at the C terminus of the H-chain constant region, but not by pre-deleting only the lysine residue at the C terminus of the H-chain constant region. All of the C-terminal sequences of the constant region of human antibodies IgG1, IgG2, and IgG4 contain lysine and glycine at positions 447 and 446, respectively, according to EU numbering (see Sequences of proteins of immunological interest, NIH Publication No.91-3242). Therefore, the method for reducing the C-terminal amino acid heterogeneity found in the present study is expected to be applicable to IgG2 and IgG4 constant regions and variants thereof.

[0155] Reduction of disulfide bond-derived heterogeneity in IgG2 isotype TOCILIZUMAB

The isotype of TOCILIZUMAB is IgG1. Since TOCILIZUMAB is a neutralizing antibody, binding to the Fc gamma receptor can be unfavorable in view of immunogenicity and adverse effects. A possible method for lowering the Fc gamma receptor binding is to convert the isotype of the IgG antibody from IgG1 to IgG2 or IgG4 (Ann Hematol. 1998 Jun; 76(6):231-48). From the viewpoint of Fc gamma receptor I binding and pharmacokinetics, IgG2 was considered to be more desirable than IgG4 (Nat Biotechnol. 2007 Dec; 25(12):1369-72). Meanwhile, physicochemical properties of proteins, in particular, homogeneity and stability are very important when developing antibodies as pharmaceuticals. The IgG2 isotype has been reported to have very high heterogeneity due to the disulfide bonds in the hinge region (J Biol Chem. 2008 Jun 6; 283(23):16206-15). It is not easy and would be more costly to manufacture them as a pharmaceutical in large-scale while maintaining the objective substances/related substances related heterogeneity derived from disulfide bonds between productions. Thus, single substances are desirable as much as possible. Thus, when developing IgG2 isotype antibodies into pharmaceuticals, it is preferable to reduce the heterogeneity derived from disulfide bonds without lowering the stability.

[0156] For the purpose of reducing the heterogeneity of the IgG2 isotype, various variants were assessed. As a result, it was found that heterogeneity could be reduced without decreasing the stability using the WT-SKSC constant region (SEQ ID NO: 70), in which of the IgG2 constant region sequences, the cysteine residue at position 131 and the arginine residue at position 133 (EU numbering) in the H-chain CH1 domain were substituted to serine and lysine, respectively, and the cysteine residue at position 219

(EU numbering) in the H-chain upper hinge was substituted to serine. TOCILIZUMAB-IgG1 (H chain WT-IgG1/SEQ ID NO: 53; L chain WT-kappa/SEQ ID NO: 54), TOCILIZUMAB-IgG2 (H chain WT-IgG2/SEQ ID NO: 71; L chain WT-kappa/SEQ ID NO: 54), and TOCILIZUMAB-SKSC (H chain WT-SKSC/SEQ ID NO: 70; L chain WT-kappa/SEQ ID NO: 54) were prepared and assessed for heterogeneity and stability. The heterogeneity was assessed by cation exchange chromatography. The ProPac WCX-10 (Dionex) column was used; and mobile phase A was 20 mM Sodium Acetate (pH 5.0) and mobile phase B was 20 mM Sodium Acetate, 1 M NaCl (pH 5.0). Appropriate flow rate and gradient were used. The assessment result obtained by cation exchange chromatography is shown in Fig. 13. The stability was assessed based on the intermediate temperature in thermal denaturation (Tm value) determined by differential scanning calorimetry (DSC) (VP-DSC; Microcal). The result of DSC measurement in 20 mM sodium acetate, 150 mM NaCl, pH 6.0 and the Tm value of the Fab domain are shown in Fig. 14.

[0157] The result showed that the heterogeneity was markedly increased in TOCILIZUMAB-IgG2 as compared to TOCILIZUMAB-IgG1; however, the heterogeneity could be significantly reduced by conversion to TOCILIZUMAB-SKSC. Furthermore, when compared to TOCILIZUMAB-IgG1, the DSC of TOCILIZUMAB-IgG2 gave a shoulder peak (Fab*) component with low stability, i.e., low Tm, in the thermal denaturation peaks of the Fab domain, which is assumed to be due to a heterogeneous component. However, when converted to TOCILIZUMAB-SKSC, the shoulder peak (low Tm), which is thought to be due to a heterogeneous component disappeared, and the Tm value was about 94 degrees C, which was equivalent to that of the Fab domain of TOCILIZUMAB-IgG1 and TOCILIZUMAB-IgG2. Thus, TOCILIZUMAB-SKSC was revealed to have high stability.

[0158] Identification of pharmacokinetics-improving mutation sites in the constant region of TOCILIZUMAB

As described above, starting from IgG1, which is the isotype of TOCILIZUMAB, reduction of the C-terminal heterogeneity and reduction of heterogeneity of antibodies with IgG2 isotype constant regions while reducing the binding to the Fc gamma receptor and maintaining the high stability can be achieved. Moreover, it is preferred that the constant region also has superior pharmacokinetics than IgG1, which is the isotype of TOCILIZUMAB.

[0159] In order to find constant regions having a superior plasma half-life than antibodies with IgG1-isotype constant regions, screening was carried out to identify mutation sites for improving the pharmacokinetics of TOCILIZUMAB-SKSC which has high stability and reduced heterogeneity related to antibodies with IgG2-isotype constant regions as mentioned above. As a result, WT-M58 (SEQ ID NO: 72 (amino acid

sequence)) was discovered, in which, as compared to WT-SKSC, the glutamic acid at position 137, EU numbering is substituted to glycine, the serine at position 138 is substituted to glycine, the histidine at position 268 is substituted to glutamine, the arginine at position 355 is substituted to glutamine, the glutamine at position 419 is substituted to glutamic acid, and in which the glycine at position 446 and the lysine at position 447 is deleted to reduce the heterogeneity of the H-chain C terminus. In addition, WT-M44 (SEQ ID NO: 73 (amino acid sequence)) was prepared to have substitution of asparagine at position 434 to alanine, relative to IgG1. Furthermore, WT-M83 (SEQ ID NO: 74 (amino acid sequence)) was produced by deleting glycine at position 446 and lysine at position 447 from M44 to reduce the heterogeneity of the H-chain C-terminus. In addition, WT-M73 (SEQ ID NO: 75 (amino acid sequence)) was produced by substituting asparagine at position 434 with alanine in WT-M58.

[0160] TOCILIZUMAB-M44 (H chain WT-M44/SEQ ID NO: 73; L chain WT-kappa/SEQ ID NO: 54), TOCILIZUMAB-M58 (H chain WT-M58/SEQ ID NO: 72; L chain WT-kappa/SEQ ID NO: 54), and TOCILIZUMAB-M73 (H chain WT-M73/SEQ ID NO: 75; L chain WT-kappa/SEQ ID NO: 54) were prepared and assessed for their affinity towards human FcRn and pharmacokinetics using human FcRn transgenic mice (see Reference Examples for the method).

[0161] The binding of TOCILIZUMAB-IgG1, TOCILIZUMAB-M44, TOCILIZUMAB-M58, and TOCILIZUMAB-M73 to human FcRn was assessed using Biacore. As shown in Table 6, the binding of TOCILIZUMAB-M44, TOCILIZUMAB-M58, and TOCILIZUMAB-M73 was about 2.7 times, 1.4 times, and 3.8 times superior than that of TOCILIZUMAB-IgG1, respectively.

[0162] [Table 6]

	KD (μM)
TOCILIZUMAB-IgG1	1.62
TOCILIZUMAB-M44	0.59
TOCILIZUMAB-M58	1.17
TOCILIZUMAB-M73	0.42

[0163] TOCILIZUMAB-IgG1, TOCILIZUMAB-M44, TOCILIZUMAB-M58, and TOCILIZUMAB-M73 were assessed for their pharmacokinetics in human FcRn transgenic mice. The result is shown in Fig. 15. When compared to TOCILIZUMAB-IgG1, all of TOCILIZUMAB-M44, TOCILIZUMAB-M58, and TOCILIZUMAB-M73 were found to exhibit improved pharmacokinetics, as shown in Fig. 15. The effect of improving the pharmacokinetics correlated with the ability to bind to human FcRn. In particular, the concentration of TOCILIZUMAB-M73 in plasma after 28 days was improved by about 16 times as compared to TOCILIZUMAB-IgG1. Thus, antibodies having the constant region of M73 were also assumed to have significantly improved

pharmacokinetics in humans as compared to antibodies having the IgG1constant region.

Example 7

[0164] Preparation of fully humanized IL-6 receptor antibodies with improved PK/PD

TOCILIZUMAB variants were prepared by combining multiple mutations in the variable and constant regions of TOCILIZUMAB found in the examples above. Fully humanized IL-6 receptor antibodies discovered from various screenings were: Fv3-M73 (H chain VH4-M73/SEQ ID NO: 25; L chain VL1-kappa/SEQ ID NO: 28), Fv4-M73 (H chain VH3-M73/SEQ ID NO: 26; L chain VL3-kappa/SEQ ID NO: 29), and Fv5-M83 (H chain VH5-M83/SEQ ID NO: 27; L chain VL5-kappa/SEQ ID NO: 30).

[0165] The affinities of prepared Fv3-M73, Fv4-M73, and Fv5-M83 against IL-6 receptor were compared to that of TOCILIZUMAB (see Reference Example for method). The affinities of these antibodies for the soluble IL-6 receptor determined at pH 7.4 are shown in Table 7. Furthermore, their BaF/gp130-neutralizing activities were compared to those of TOCILIZUMAB and the control (the known high affinity anti-IL-6 receptor antibody described in Reference Example, and VQ8F11-21 hIgG1 described in US 2007/0280945) (see Reference Example for method). The results obtained by determining the biological activities of these antibodies using BaF/gp130 are shown in Fig. 16 (TOCILIZUMAB, the control, and Fv5-M83 with a final IL-6 concentration of 300 ng/ml) and Fig. 17 (TOCILIZUMAB, Fv3-M73, and Fv4-M73 with a final IL-6 concentration of 30 ng/ml). As shown in Table 7, Fv3-M73 and Fv4-M73 have about two to three times higher affinity than TOCILIZUMAB, while Fv5-M83 exhibits about 100 times higher affinity than TOCILIZUMAB (since it was difficult to measure the affinity of Fv5-M83, instead the affinity was determined using Fv5-IgG1 (H chain VH5-IgG1 /SEQ ID NO: 76; L chain VL5-kappa /SEQ ID NO: 30), which has an IgG1-type constant region; the constant region is generally thought to have no effect on affinity). As shown in Fig. 17, Fv3-M73 and Fv4-M73 exhibit slightly stronger activities than TOCILIZUMAB. As shown in Fig. 16, Fv5-M83 has a very strong activity, which is more than 100 times greater than that of TOCILIZUMAB in terms of 50% inhibitory concentration. Fv5-M83 also exhibits about 10 times higher neutralizing activity in terms of 50% inhibitory concentration than the control (the known high-affinity anti-IL-6 receptor antibody).

[0166] [Table 7]

	k_a(1/Ms)	k_d(1/s)	KD (M)
TOCILIZUMAB	4.0E+05	1.1E-03	2.7E-09
Fv3-M73	8.5E+05	8.7E-04	1.0E-09
Fv4-M73	7.5E+05	1.0E-03	1.4E-09
Fv5-M83	1.1E+06	2.8E-05	2.5E-11

[0167] The rates of dissociation of TOCILIZUMAB, Fv3-M73, and Fv4-M73 from membrane-type IL-6 receptor at pH 7.4 and 5.8 were determined. As demonstrated by the result shown in Table 8 (see Reference Example for method), the pH dependency of the dissociation rate of Fv3-M73 and Fv4-M73 from membrane-type IL-6 receptor was about 11 times and 10 times improved, respectively, as compared to TOCILIZUMAB. The considerable improvement of the pH dependency of the dissociation rate relative to H3pI/L73 described in Example 5 suggested that when compared to H3pI/L73, pharmacokinetics of Fv3-M73 and Fv4-M73 would be significantly improved.

[0168] [Table 8]

	pH7.4 k_d(1/s)	pH5.8 k_d(1/s)	$k_{d(pH5.8)} / k_{d(pH7.4)}$ pH DEPENDENCY
TOCILIZUMAB	2.5E-04	2.5E-04	1.00
Fv3-M73	4.9E-04	5.3E-03	10.88
Fv4-M73	5.1E-04	5.1E-03	10.06

[0169] The isoelectric points of TOCILIZUMAB, the control, Fv3-M73, Fv4-M73, and Fv5-M83 were determined by isoelectric focusing electrophoresis using a method known to those skilled in the art. The result showed that the isoelectric point was about 9.3 for TOCILIZUMAB; about 8.4 to 8.5 for the control; about 5.7 to 5.8 for Fv3-M73; about 5.6 to 5.7 for Fv4-M73; and 5.4 to 5.5 for Fv5-M83. Thus, each antibody had a significantly lowered isoelectric point when compared to TOCILIZUMAB and the control. Furthermore, the theoretical isoelectric point of the variable regions VH/VL was calculated by GENETYX (GENETYX CORPORATION). The result showed that the theoretical isoelectric point was 9.20 for TOCILIZUMAB; 7.79 for the control; 5.49 for Fv3-M73; 5.01 for Fv4-M73; and 4.27 for Fv5-M83. Thus, each antibody had a significantly lowered isoelectric point when compared to TOCILIZUMAB and the control. Since it was shown in Example 2 that pharmacokinetics is improved by reducing the isoelectric point, the pharmacokinetics of Fv3-M73, Fv4-M73, and Fv5-M83 was thought to be improved when compared to TOCILIZUMAB and the control.

[0170] T-cell epitopes in the variable region sequence of TOCILIZUMAB, Fv3-M73, Fv4-M73, or Fv5-M83 were analyzed using TEPITOPE (Methods. 2004 Dec;34(4):468-75). As a result, TOCILIZUMAB was predicted to have T-cell epitopes, of which many could bind to HLA, as shown in Example 3. In contrast, the number of sequences that were predicted to bind to T-cell epitopes was significantly reduced in Fv3-M73, Fv4-M73, and Fv5-M83. In addition, the framework of Fv3-M73, Fv4-M73, or Fv5-M83 has no mouse sequence and is thus fully humanized. These suggest the possibility that the immunogenicity risk is significantly reduced in

Fv3-M73, Fv4-M73, and Fv5-M83 when compared to TOCILIZUMAB.

Example 8

[0171] PK/PD test of fully humanized IL-6 receptor antibodies in monkeys

Each of TOCILIZUMAB, the control, Fv3-M73, Fv4-M73, and Fv5-M83 was intravenously administered once at a dose of 1 mg/kg to cynomolgus monkeys to assess their time course of plasma concentration (see Reference Example for method). The plasma concentration time courses of TOCILIZUMAB, Fv3-M73, Fv4-M73, and Fv5-M83 after intravenous administration are shown in Fig. 18. The result showed that each of Fv3-M73, Fv4-M73, and Fv5-M83 exhibited significantly improved pharmacokinetics in cynomolgus monkeys when compared to TOCILIZUMAB and the control. Of them, Fv3-M73 and Fv4-M73 exhibited highly improved pharmacokinetics when compared to TOCILIZUMAB.

[0172] The efficacy of each antibody to neutralize membrane-type cynomolgus monkey IL-6 receptor was assessed. Cynomolgus monkey IL-6 was administered subcutaneously in the lower back at 5 microgramme/kg every day from Day 6 to Day 18 after antibody administration (Day 3 to Day 10 for TOCILIZUMAB), and the CRP concentration in each animal was determined 24 hours later (see Reference Example for method). The time course of CRP concentration after administration of each antibody is shown in Fig. 19. To assess the efficacy of each antibody to neutralize soluble cynomolgus monkey IL-6 receptor, the plasma concentration of free soluble cynomolgus monkey IL-6 receptor in the cynomolgus monkeys was determined and the percentages of free soluble IL-6 receptor were calculated (see Reference Example for method). The time course of percentage of free soluble IL-6 receptor after administration of each antibody is shown in Fig. 20.

[0173] Each of Fv3-M73, Fv4-M73, and Fv5-M83 neutralized membrane-type cynomolgus monkey IL-6 receptor in a more sustainable way, and suppressed the increase of CRP over a longer period when compared to TOCILIZUMAB and the control (the known high-affinity anti-IL-6 receptor antibody). Furthermore, each of Fv3-M73, Fv4-M73, and Fv5-M83 neutralized soluble cynomolgus monkey IL-6 receptor in a more sustainable way, and suppressed the increase of free soluble cynomolgus monkey IL-6 receptor over a longer period when compared to TOCILIZUMAB and the control. These findings demonstrate that all of Fv3-M73, Fv4-M73, and Fv5-M83 are superior in sustaining the neutralization of membrane-type and soluble IL-6 receptors than TOCILIZUMAB and the control. Of them, Fv3-M73 and Fv4-M73 are remarkably superior in sustaining the neutralization. Meanwhile, Fv5-M83 suppressed CRP and free soluble cynomolgus monkey IL-6 receptor more strongly than Fv3-M73 and Fv4-M73. Thus, Fv5-M83 is considered to be stronger than Fv3-M73, Fv4-M73, and

the control (the known high-affinity anti-IL-6 receptor antibody) in neutralizing membrane-type and soluble IL-6 receptors. It was considered that results in in vivo of cynomolgus monkeys reflect the stronger affinity of Fv5-M83 for IL-6 receptor and stronger biological activity of Fv5-M83 in the BaF/gp130 assay system relative to the control.

[0174] These findings suggest that Fv3-M73 and Fv4-M73 are highly superior in sustaining their activities as an anti-IL-6 receptor-neutralizing antibody when compared to TOCILIZUMAB and the control, and thus enable to significantly reduce the dosage and frequency of administration. Furthermore, Fv5-M83 was demonstrated to be remarkably superior in terms of the strength of activity as an anti-IL-6 receptor-neutralizing antibody as well as sustaining their activity. Thus, Fv3-M73, Fv4-M73, and Fv5-M83 are expected to be useful as pharmaceutical IL-6 antagonists.

Example 9

[0175] Selection of the stable buffer species for Fv4-M73

Fv4-M73 is prepared as described above. Since Fv4-M73 is composed of non-natural constant region M73 for improved heterogeneity, stability, safety, and pharmacokinetics, as is described in Examples 6-7, stability profile (such as most stable buffer species) of Fv4-M73 might be different from antibody composed of natural constant region such as IgG1. It is reported that antibody composed of natural IgG1 constant region is generally most stable under histidine-acetate buffer (WO/2006/044908). So the effect of buffer species on stability of Fv4-M73 was tested.

[0176] Fv4-M73 was formulated to a final concentration of 37 mg/mL in four different buffer formulations (as described in Table 9) having a pH of 6.0. The samples were analyzed by size exclusion chromatography and anion exchange chromatography with UV detection over a two-month period under storage conditions at 40 degrees C. The percentage of aggregates formed in these formulations is displayed over time and is shown in Fig. 21. A percentage increase of aggregates is an indication for a reduced stability of the antibody. Therefore, the percentage increase has been used as indicator to compare the stabilization effect of the different buffers.

[0177] As shown in Fig. 21, the formulations with histidine-HCl buffer (formulation A) and the citrate buffer (formulation D) were the most stable, and the formulation with the acetate buffer (formulation C) was the most unstable. The histidine-acetate buffer (formulation B) was slightly more unstable than histidine-HCl buffer which is assumed to be due to destablizing effect of acetate buffer.

[0178] Thus it was found that Fv4-M73 with non-natural constant region is the most stable in histidine-HCl buffer and citrate buffer, not in histidine-acetate buffer as is reported to be the most stable buffer species for natural IgG1 antibody.

[0179] [Table 9] Formulations used in Example 9

mAb	Conc. mg/mL	Formulation	Buffer	NaCl	pH
Fv4-M73	37	A	20 mM Histidine-HCl	150 mM	6.0
		B	20 mM Histidine-acetate		
		C	20 mM Acetate		
		D	20 mM Citrate		

[0180] Methods

Size Exclusion Chromatography (SEC): Size exclusion chromatography was performed to screen the antibody formulation for the presence of antibody aggregates and fragments. The samples were injected on a size exclusion G3000 SW_{XL} column (TOSOH). The mobile phase was 50 mM sodium phosphate, 300 mM sodium chloride (pH 7.0), running isocratically with a flow rate of 0.5 mL/min. The eluted protein was detected by UV absorbance at 220 nm. The relative amount of any protein species detected was reported as the area percent of the product peak, compared to the total area of all other detected peaks. Peaks eluting earlier than the antibody monomer peak were recorded in the aggregates percentile, while peaks eluting later than the antibody monomer peak, but earlier than the buffer peak, were recorded in the fragments percentile.

[0181] Sample preparation: Samples were diluted to 0.3-2 mg/mL with mobile phase, and 15 microliter were injected to the column.

[0182] Anion exchange chromatography: Anion exchange chromatography was performed to analyze the antibody formulation for the presence of heterogeneity, especially for the presence of deamidation (ones with deamidation being eluted as acidic species after the main peak). The samples were injected onto a DEAE-NPR column (TOSOH) at 40 degrees C. The mobile phase was A) 10 mM Tris-HCl (pH 7.5), B) 10 mM Tris-HCl, 500 mM sodium chloride (pH 7.5), running with a gradient of 100% mobile phase A, and then 70% mobile phase A and 30% mobile phase B in 30 min, followed by 100% mobile phase B in 1 min, which was then maintained for 4 min to wash the column at a flow rate of 1.0 mL/min. Eluted protein was detected by UV absorbance at 280 nm.

[0183] Sample preparation: Samples were diluted 0.05-0.33 mg/mL with the mobile phase, and inject in a volume of 100 microliter to the column.

Example 10

[0184] Effects of pH, NaCl, and Arginine-HCl on stability of Fv4-M73 at 100 mg/mL.

Fv4-M73 was formulated to a final concentration of 100 mg/mL in different buffer formulations (described in Table 10), and the samples were analyzed by size exclusion chromatography and anion exchange chromatography with UV detection over a two-month period when stored at 25 degrees C and 40 degrees C, and also on freeze-thawed samples (freezing for 0.5 day at -20 degrees C, and thawing for 30 min at room

temperature). The percentage of aggregates present in the formulations and percentage of aggregates increased from initial after two/four/eight-week storage at 25 degrees C and 40 degrees C is shown in Figs. 22-25. An increase of the amount of aggregates is indicative for a reduced stability.

[0185] [Table 10] Formulations used in Example 10

Buffer		20mM Histidine-HCl			20mM Citrate		
NaCl		50mM	150mM	50mM	50mM	150mM	50mM
Arginine-HCl		-	-	100mM	-	-	100mM
Target pH	4.5	*4.3*	*4.3*	*4.0*	*4.3*	*4.3*	*4.5*
	5.0	*4.9*	*4.9*	*5.0*	*4.8*	*4.9*	*4.8*
	5.5	*5.4*	*5.4*	*5.4*	*5.4*	*5.4*	*5.4*
	6.0	*6.0*	*5.8*	*5.8*	*6.0*	*5.9*	*5.7*
	6.5	*6.4*	*6.3*	*6.3*	*6.2*	*6.2*	*6.2*
	7.0	*6.6*	*6.7*	*6.6*	*6.5*	-	*6.8*

Actual value (pH)

[0186] Low molecular weight species (LMW) described in Fig. 26 are assumed to be the result of a direct hydrolysis of peptide bonds on the weak spots such as the hinge region under acidic or basic conditions. This is especially common for monoclonal antibodies in liquid formulations at high temperatures. Acidic species eluted from about 22.5 min to 26 min, which increased under basic conditions and described in Fig. 27, are assumed to be a result of non enzymatic deamidation of asparagine residues (a common modification of antibodies), which has been reported to be involved in charge heterogeneity of monoclonal antibodies by observing more acidic species after incubating antibodies under basic pH at elevated temperatures. Considering the results of Figs. 22-26, Fv4-M73 was found to be stable in solution at a pH from about 5.5 to 6.3 with regard to aggregates and degradation. An optimal stability was observed at a pH from about 5.5 to 6.3 with regard to the main peak ratio to the total peak area (Figs. 27-28).

[0187] Within this study, adding 100 mM NaCl to the buffer containing 20 mM buffering agent and 50 mM NaCl (20 mM buffer, 150 mM NaCl) led to an equally stable antibody solution as the buffer containing 20 mM buffering agent and 50 mM NaCl (20 mM buffer, 50 mM NaCl), suggesting that NaCl has no stabilizing effect. While adding 100 mM arginine to the buffer containing 20 mM buffering agent and 50 mM NaCl (20 mM buffer, 50 mM NaCl, and 100 mM arginine-HCl) showed a significant stabilizing effect of the antibody solution with respect to aggregates, suggesting that arginine-HCl has a significant stabilizing effect. Regarding buffering agents, the solution stability was slightly better using histidine-HCl buffer than citrate buffer.

[0188] Regarding the freeze-thawing study ("FT" indicates the number of freeze/thaw

cycles) as shown in Fig. 29, a significant amount of aggregates was observed at a lower pH or lower salt concentration in the formulations. Adding 100 mM arginine to the buffer containing 20 mM buffering agent and 50 mM NaCl (20 mM buffer, 50 mM NaCl, 100 mM arginine-HCl) showed to be more effective with regard to the formation of aggregates than adding 100 mM NaCl (20 mM buffer, 150 mM NaCl), suggesting that arginine-HCl has a significant stabilizing effect against free-thaw. Optimal stability was observed at a pH ranging from about 5.0 to 6.6 containing 100 mM arginine-HCl.

Example 11

[0189] Effect of sugar and Arginine-HCl on stability of Fv4-M73 at 100mg/mL.

Fv4-M73 was formulated to a final concentration of 100 mg/mL in different buffer formulations (described Table 11), and the samples were analyzed by size exclusion chromatography and anion exchange chromatography with UV detection over a two-month period of time when stored at 25 degrees C and 40 degrees C, and also subjected a specified number of freeze/thaw cycles (freezing for 0.5 day at -20 degrees C, and thawing for 30 min at room temperature). The percentage of aggregates contained in the formulations increased from initial are plotted over time and shown in Figs. 30 and 31. An increase of the amount of aggregates in percent is indicative for a reduced stability.

[0190] As shown in Figs. 30 and 31, formulations F and G provided less stability than formulation H, showing sucrose and trehalose have less stabilizing effect than arginine-HCl in liquid condition stored at 25 degrees C and 40 degrees C, while in freeze-thawing, formulations F and G provided comparable or more stability than formulation H, showing that sucrose or trehalose showed significant stabilizing effects in freeze-thawing.

[0191] [Table 11] Formulations used in Example 11

mAb	Conc. mg/mL	Formulation	Excipient	Buffer	pH
Fv4-M73	100	E	None	20 mM Histidine-HCl, 50 mM NaCl	6.5
		F	100 mM Sucrose		
		G	100 mM Trehalose		
		H	100 mM Arginine-HCl		

Example 12

[0192] Stability of Fv4-M73 at 200 mg/mL; effect of pH, Arginine-HCl and trehalose

Fv4-M73 was formulated to a final concentration of 200 mg/mL in six different formulations as described in Table 12.

[0193] [Table 12] Formulations used in Example 12

mAb	Conc. mg/mL	Formulation	Buffer	Arg-HCl (mM)	excipient	pH
Fv4-M73	200	1	20 mM Histidine	100	-	5.5
		2				6.0
		3				6.5
		4		50		6.0
		5		150		
		6		100	50 mM Trehalose	

[0194] The samples were incubated at 5 degrees C and -20 degrees C for three months and six months respectively. Initial samples, three months samples and six months samples were analyzed by size exclusion chromatography with UV detection as described in Example 9. The percentage of aggregates increased at 5 degrees C from initial in these formulations after three months and six months is shown in Fig. 32. The percentage of aggregates increased from initial at -20 degrees C in these formulations after three months and six months is shown in Fig. 33.

[0195] As shown in Fig. 32, in liquid condition stored at 5 degrees C, Fv4-M73 was apparently more stable at lower pH (most stable at pH 5.5 and least stable at pH 6.5) and more stable at higher arginine concentration (most stable at 150 mM arginine-HCl and least stable at 50 mM arginine-HCl). Addition of 50 mM trehalose showed some stabilizing effect on Fv4-M73 in liquid condition stored at 5 degrees C. Since pharmaceutical liquid formulations of antibodies are often stored at 5 degrees C, preferable liquid formulation for Fv4-M73 should contain at least 100 mM arginine-HCl with histidine buffer pH 5.5 to pH 6.0 and additional stabilizer if necessary.

[0196] As shown in Fig. 33, in frozen condition stored at -20 degrees C, Fv4-M73 tends to be more stable at higher pH and was apparently more stable at higher arginine concentration (most stable at 150 mM arginine-HCl and least stable at 50 mM arginine-HCl). Addition of 50 mM trehalose showed significant stabilizing effect on Fv4-M73 in frozen condition stored at -20 degrees C. Bulk substance of antibody is often stored in frozen state at -20 degrees C to -70 degrees C. Considering the cost of the storage and shipping at frozen state, -20 degrees C storage is desirable. Preferable bulk substance formulation for -20 degrees C storage of Fv4-M73 should contain at least 100 mM arginine-HCl with histidine buffer pH 5.5 to pH 6.5 and sugar (such as trehalose).

[0197] Reference Examples

Preparation of soluble recombinant human IL-6 receptor

Soluble recombinant human IL-6 receptor of the human IL-6 receptor, which is the antigen, was produced as described below. A CHO cell line constitutively expressing a

soluble human IL-6 receptor containing a sequence from the N-terminal 1st to 344th amino acids reported in J. Biochem. (1990) 108, 673-676 (Yamasaki et al., Science (1988) 241, 825-828 (GenBank #X12830)) was generated. Soluble human IL-6 receptor was purified from culture supernatant of CHO cells expressing SR344 by three column chromatographies: Blue Sepharose 6 FF column chromatography, affinity chromatography using a column immobilized with an antibody specific to SR344, and gel filtration column chromatography. The fraction eluted as the main peak was used as the final purified sample.

[0198] Preparation of soluble recombinant cynomolgus monkey IL-6 receptor (cIL-6R)

Oligo-DNA primers were prepared based on the disclosed gene sequence for Rhesus monkey IL-6 receptor (Birney et al., Ensembl 2006, Nucleic Acids Res. 2006 Jan 1;34 (Database issue):D556-61). A DNA fragment encoding the whole cynomolgus monkey IL-6 receptor gene was prepared by PCR using the primers, and as a template, cDNA prepared from the pancreas of cynomolgus monkey. The resulting DNA fragment was inserted into a mammalian cell expression vector, and a stable expression CHO line (cyno.sIL-6R-producing CHO cell line) was prepared using the vector. The culture medium of cyno.sIL-6R-producing CHO cells was purified using a HisTrap column (GE Healthcare Bioscience) and then concentrated with Amicon Ultra-15 Ultracel-10k (Millipore). A final purified sample of soluble cynomolgus monkey IL-6 receptor (hereinafter cIL-6R) was obtained through further purification on a Superdex200pg16/60 gel filtration column (GE Healthcare Bioscience).

[0199] Preparation of recombinant cynomolgus monkey IL-6 (cIL-6)

Cynomolgus monkey IL-6 was prepared by the procedure described below. The nucleotide sequence encoding 212 amino acids deposited under SWISSPROT Accession No. P79341 was prepared and cloned into a mammalian cell expression vector. The resulting vector was introduced into CHO cells to prepare a stable expression cell line (cyno.IL-6-producing CHO cell line). The culture medium of cyno.IL-6-producing CHO cells was purified using a SP-Sepharose/FF column (GE Healthcare Bioscience) and then concentrated with Amicon Ultra-15 Ultracel-5k (Millipore). A final purified sample of cynomolgus monkey IL-6 (hereinafter cIL-6) was obtained through further purification on a Superdex75pg26/60 gel filtration column (GE Healthcare Bioscience), followed by concentration with Amicon Ultra-15 Ultracel-5k (Millipore).

[0200] Preparation of a known high-affinity anti-IL-6 receptor antibody

A mammalian cell expression vector was constructed to express VQ8F11-21 hIgG1, a known high-affinity anti-IL-6 receptor antibody. VQ8F11-21 hIgG1 is described in US 2007/0280945 A1 (US 2007/0280945 A1; the amino acid sequences of H chain and L chain as set forth in SEQ ID NOs: 77 and 78, respectively). The antibody variable region was constructed by PCR using a combination of synthetic oligo DNAs

(assembly PCR) and IgG1 was used for the constant region. The antibody variable and constant regions were combined together by assembly PCR, and then inserted into a mammalian expression vector to construct expression vectors for the H chain and L chain of interest. The nucleotide sequences of the resulting expression vectors were determined by a method known to those skilled in the art. The high-affinity anti-IL-6 receptor antibody (hereinafter abbreviated as "control") was expressed and purified using the constructed expression vectors by the method described in Example 1.

[0201] Preparation, expression, and purification of TOCILIZUMAB variants

TOCILIZUMAB variants were prepared using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the method described in the appended instruction manual. The resulting plasmid fragments were inserted into mammalian cell expression vectors to construct expression vectors for the H chains and L chains of interest. The nucleotide sequences of the obtained expression vectors were determined by a method known to skilled artisans. The antibodies were expressed by the method described below. Human embryonic kidney cancer-derived HEK293H cell line (Invitrogen) was suspended in DMEM (Invitrogen) supplemented with 10% Fetal Bovine Serum (Invitrogen). The cells were plated at 10 ml per dish in dishes for adherent cells (10 cm in diameter; CORNING) at a cell density of 5 to 6 x 10^5 cells/ml and cultured in a CO_2 incubator (37 degrees C, 5% CO_2) for one whole day and night. Then, the medium was removed by aspiration, and 6.9 ml of CHO-S-SFM-II medium (Invitrogen) was added. The prepared plasmid was introduced into the cells by the lipofection method. The resulting culture supernatants were collected, centrifuged (approximately 2000 g, 5 min, room temperature) to remove cells, and sterilized by filtering through 0.22-micrometer filter MILLEX(R)-GV (Millipore) to obtain the supernatants. Antibodies were purified from the obtained culture supernatants by a method known to those skilled in the art using rProtein A Sepharose™ Fast Flow (Amersham Biosciences). To determine the concentration of the purified antibody, absorbance was measured at 280 nm using a spectrophotometer. Antibody concentrations were calculated from the determined values using an absorbance coefficient calculated by the PACE method (Protein Science 1995; 4:2411-2423).

[0202] Establishment of a human gp130-expressing BaF3 cell line

A BaF3 cell line expressing human gp130 was established by the procedure described below to obtain a cell line that proliferates in an IL-6-dependent manner.

[0203] A full-length human gp130 cDNA (Hibi et al., Cell (1990) 63:1149-1157 (GenBank #NM_002184)) was amplified by PCR and cloned into the expression vector pCOS2Zeo to construct pCOS2Zeo/gp130. pCOS2Zeo is an expression vector constructed by removing the DHFR gene expression region from pCHOI (Hirata et al., FEBS Letter (1994) 356:244-248) and inserting the expression region of the Zeocin re-

sistance gene. The full-length human IL-6R cDNA was amplified by PCR and cloned into pcDNA3.1(+) (Invitrogen) to construct hIL-6R/pcDNA3.1(+).

[0204] 10 microgramme of pCOS2Zeo/gp130 was mixed with BaF3 cells (0.8 x 10^7 cells) suspended in PBS, and then pulsed at 0.33 kV and 950 micro FD using Gene Pulser (Bio-Rad). The BaF3 cells having the gene introduced by electroporation were cultured for one whole day and night in RPMI 1640 medium (Invitrogen) supplemented with 0.2 ng/ml mouse interleukin-3 (Peprotech) and 10% fetal bovine serum (hereinafter FBS, HyClone), and selected by adding RPMI 1640 medium supplemented with 100 ng/ml human interleukin-6 (R&D systems), 100 ng/ml human interleukin-6 soluble receptor (R&D systems), and 10% FBS to establish a human gp130-expressing BaF3 cell line (hereinafter "BaF3/gp130"). This BaF/gp130 proliferates in the presence of human interleukin-6 (R&D systems) and soluble human IL-6 receptor, and thus can be used to assess the growth inhibition activity (or IL-6 receptor neutralizing activity) of an anti-IL-6 receptor antibody.

[0205] Assessment for the biological activity by human gp130-expressing BaF3 cells (BaF/gp130)

The IL-6 receptor neutralizing activity was assessed using BaF3/gp130 which proliferates in an IL-6/IL-6 receptor-dependent manner. After three washes with RPMI1640 supplemented with 10% FBS, BaF3/gp130 cells were suspended at 5 x 10^4 cells/ml in RPMI1640 supplemented with 600 ng/ml or 60 ng/ml human interleukin-6 (TORAY) (final concentration of 300 ng/ml or 30 ng/ml), appropriate amount of soluble human IL-6 receptor, and 10% FBS. The cell suspensions were dispensed (50 microliter/well) into 96-well plates (CORNING). Then, the purified antibodies were diluted with RPMI1640 containing 10% FBS, and added to each well (50 microliter/well). The cells were cultured at 37 degrees C under 5% CO_2 for three days. WST-8 Reagent (Cell Counting Kit-8; Dojindo Laboratories) was diluted two-fold with PBS. Immediately after 20 microliter of the reagent was added to each well, the absorbance at 450 nm (reference wavelength: 620 nm) was measured using SUNRISE CLASSIC (TECAN). After culturing for two hours, the absorbance at 450 nm (reference wavelength: 620 nm) was measured again. The IL-6 receptor neutralizing activity was assessed using the change of absorbance during two hours as an indicator.

[0206] Biacore-based analysis of binding to soluble human IL-6 receptor

Antigen-antibody reaction kinetics was analyzed using Biacore T100 (GE Healthcare). The soluble human IL-6 receptor-antibody interaction was measured by immobilizing appropriate amounts of protein A or protein A/G or anti-IgG (gamma-chain specific) $F(ab')_2$ onto a sensor chip by amine coupling method, binding antibodies of interest onto the chip at pH7.4, and then running soluble IL-6 receptor adjusted to various concentrations at pH7.4 over the chip as an analyte. All mea-

surements were carried out at 37 degrees C. The kinetic parameters, association rate constant k_a (1/Ms) and dissociation rate constant k_d (1/s) were calculated from the sensorgrams obtained by measurement. Then, K_D (M) was determined based on the rate constants. The respective parameters were determined using Biacore T100 Evaluation Software (GE Healthcare).

[0207] Assessment for the pH-dependent dissociation from membrane-type IL-6 receptor using Biacore

The antigen-antibody reaction with membrane-type IL-6 receptor at pH 5.8 and pH 7.4 was observed using Biacore T100 (GE Healthcare). The binding to membrane-type IL-6 receptor was assessed by evaluating the binding to soluble human IL-6 receptor immobilized onto the sensor chip. SR344 was biotinylated by a method known to those skilled in the art. Based on the affinity between biotin and streptavidin, biotinylated soluble human IL-6 receptor was immobilized onto the sensor chip via streptavidin. All measurements were conducted at 37 degrees C. The mobile phase buffer was 10 mM MES (pH 5.8), 150 mM NaCl, and 0.05% Tween 20. A clone exhibiting pH-dependent binding was injected under the condition of pH 7.4 to bind to soluble human IL-6 receptor (injection sample buffer was 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20). Then, the pH-dependent dissociation of each clone was observed at pH 5.8, which is the pH of the mobile phase. The dissociation rate constant (kd (1/s)) at pH 5.8 was calculated using Biacore T100 Evaluation Software (GE Healthcare) by fitting only the dissociation phase at pH 5.8. The sample concentration was 0.25 microgramme/ml. Binding was carried out in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, and dissociation was carried out in 10 mM MES (pH 5.8), 150 mM NaCl, and 0.05% Tween 20. Likewise, the dissociation rate constant (kd (1/s)) at pH 7.4 was calculated using Biacore T100 Evaluation Software (GE Healthcare) by fitting only the dissociation phase at pH 7.4. The sample concentration was 0.5 microgramme/ml. Binding was carried out in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, and dissociation was carried out in 10 mM MES (pH 7.4), 150 mM NaCl, and 0.05% Tween 20.

[0208] Assessment of the binding to human FcRn

FcRn is a complex of FcRn and beta 2-microglobulin. Oligo-DNA primers were prepared based on the human FcRn gene sequence disclosed (J. Exp. Med. (1994) 180(6):2377-2381). A DNA fragment encoding the whole gene was prepared by PCR using human cDNA (Human Placenta Marathon-Ready cDNA, Clontech) as a template and the prepared primers. Using the obtained DNA fragment as a template, a DNA fragment encoding the extracellular domain containing the signal region (Met1-Leu290) was amplified by PCR, and inserted into an animal cell expression vector (the amino acid sequence of human FcRn as set forth in SEQ ID NO: 79).

Likewise, oligo-DNA primers were prepared based on the human beta 2-microglobulin gene sequence disclosed (Proc. Natl. Acad. Sci. USA. (2002) 99(26):16899-16903). A DNA fragment encoding the whole gene was prepared by PCR using human cDNA (Hu-Placenta Marathon-Ready cDNA, CLONTECH) as a template and the prepared primers. Using the obtained DNA fragment as a template, a DNA fragment encoding the whole beta 2-microglobulin containing the signal region (Met1-Met119) was amplified by PCR and inserted into a mammalian cell expression vector (the amino acid sequence of human beta 2-microglobulin as set forth in SEQ ID NO: 80).

[0209] Soluble human FcRn was expressed by the following procedure. The plasmids constructed for human FcRn and beta 2-microglobulin were introduced into cells of the human embryonic kidney cancer-derived cell line HEK293H (Invitrogen) using 10% FBS (Invitrogen) by lipofection. The resulting culture supernatant was collected, and FcRn was purified using IgG Sepharose 6 Fast Flow (Amersham Biosciences) by the method described in J. Immunol. 2002 Nov 1;169(9):5171-80, followed by further purification using HiTrap Q HP (GE Healthcare).

[0210] Determination of antibody concentration in mouse plasma

Antibody concentrations in mouse plasma were determined by ELISA according to a method known to those skilled in the art.

[0211] PK/PD test to determine the antibody concentration in the plasma, CRP concentration, and free soluble IL-6 receptor in monkeys

The plasma concentrations in cynomolgus monkeys were determined by ELISA using a method known to those skilled in the art.

[0212] The concentration of CRP was determined with an automated analyzer (TBA-120FR; Toshiba Medical Systems Co.) using Cias R CRP (KANTO CHEMICAL CO., INC.).

[0213] The plasma concentration of free soluble cynomolgus monkey IL-6 receptor in cynomolgus monkeys was determined by the procedure described below. All IgG-type antibodies (cynomolgus monkey IgG, anti-human IL-6 receptor antibody, and anti-human IL-6 receptor antibody-soluble cynomolgus monkey IL-6 receptor complex) in the plasma were adsorbed onto Protein A by loading 30 microliter of cynomolgus monkey plasma onto an appropriate amount of rProtein A Sepharose Fast Flow resin (GE Healthcare) dried in a 0.22-micrometer filter cup (Millipore). Then, the solution in cup was spinned down using a high-speed centrifuge to collect the solution that passed through. The solution that passed through does not contain Protein A-bound anti-human IL-6 receptor antibody-soluble cynomolgus monkey IL-6 receptor complex. Therefore, the concentration of free soluble IL-6 receptor can be determined by measuring the concentration of soluble cynomolgus monkey IL-6 receptor in the solution that passed through Protein A. The concentration of soluble cynomolgus monkey IL-6 receptor was determined using a method known to those skilled in the art

for measuring the concentrations of soluble human IL-6 receptor. Soluble cynomolgus monkey IL-6 receptor (cIL-6R) prepared as described above was used as a standard. The percentage of free soluble IL-6 receptor was calculated by the following formula.

$$\frac{\text{Free soluble IL-6 receptor concentration after antibody administration}}{\text{Soluble IL-6 receptor concentration before antibody administration}} \times 100$$

Claims

[Claim 1] A pharmaceutical formulation, comprising at least one polypeptide selected from:
(a) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 1 (CDR1 of VH4-M73), CDR2 comprising the sequence of SEQ ID NO: 2 (CDR2 of VH4-M73), and CDR3 comprising the sequence of SEQ ID NO: 3 (CDR3 of VH4-M73);
(b) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 4 (CDR1 of VH3-M73), CDR2 comprising the sequence of SEQ ID NO: 5 (CDR2 of VH3-M73), and CDR3 comprising the sequence of SEQ ID NO: 6 (CDR3 of VH3-M73);
(c) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 7 (CDR1 of VH5-M83), CDR2 comprising the sequence of SEQ ID NO: 8 (CDR2 of VH5-M83), and CDR3 comprising the sequence of SEQ ID NO: 9 (CDR3 of VH5-M83);
(d) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 10 (CDR1 of VL1), CDR2 comprising the sequence of SEQ ID NO: 11 (CDR2 of VL1), and CDR3 comprising the sequence of SEQ ID NO: 12 (CDR3 of VL1);
(e) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 13 (CDR1 of VL3), CDR2 comprising the sequence of SEQ ID NO: 14 (CDR2 of VL3), and CDR3 comprising the sequence of SEQ ID NO: 15 (CDR3 of VL3); and
(f) a polypeptide that comprises CDR1 comprising the sequence of SEQ ID NO: 16 (CDR1 of VL5), CDR2 comprising the sequence of SEQ ID NO: 17 (CDR2 of VL5), and CDR3 comprising the sequence of SEQ ID NO: 18 (CDR3 of VL5).

[Claim 2] A pharmaceutical formulation, comprising at least one antibody selected from:
(a) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 1 (CDR1 of VH4-M73), CDR2 comprising the sequence of SEQ ID NO: 2 (CDR2 of VH4-M73), and CDR3 comprising the sequence of SEQ ID NO: 3 (CDR3 of VH4-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 10 (CDR1 of VL1), CDR2 comprising the sequence of SEQ ID NO: 11 (CDR2 of VL1), and CDR3 comprising the sequence of SEQ ID NO: 12 (CDR3 of

VL1);
(b) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 4 (CDR1 of VH3-M73), CDR2 comprising the sequence of SEQ ID NO: 5 (CDR2 of VH3-M73), and CDR3 comprising the sequence of SEQ ID NO: 6 (CDR3 of VH3-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 13 (CDR1 of VL3), CDR2 comprising the sequence of SEQ ID NO: 14 (CDR2 of VL3), and CDR3 comprising the sequence of SEQ ID NO: 15 (CDR3 of VL3); and
(c) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 7 (CDR1 of VH5-M83), CDR2 comprising the sequence of SEQ ID NO: 8 (CDR2 of VH5-M83), and CDR3 comprising the sequence of SEQ ID NO: 9 (CDR3 of VH5-M83), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 16 (CDR1 of VL5), CDR2 comprising the sequence of SEQ ID NO: 17 (CDR2 of VL5), and CDR3 comprising the sequence of SEQ ID NO: 18 (CDR3 of VL5).
(d) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 19 (variable region of VH4-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 22 (variable region of VL1);
(e) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 20 (variable region of VH3-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 23 (variable region of VL3); and
(f) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 (variable region of VH5-M83) and a light chain variable region comprising the sequence of SEQ ID NO: 24 (variable region of VL5).
(g) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 25 (VH4-M73) and a light chain comprising the sequence of SEQ ID NO: 28 (VL1);
(h) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 26 (VH3-M73) and a light chain comprising the sequence of SEQ ID NO: 29 (VL3); and
(i) an antibody that comprises a heavy chain comprising the sequence

of SEQ ID NO: 27 (VH5-M83) and a light chain comprising the sequence of SEQ ID NO: 30 (VL5).

[Claim 3] A stable pharmaceutical formulation according to claim 1 or 2, comprising a histidine and/or citrate buffer.

[Claim 4] A stable pharmaceutical formulation according to any one of claims 1-3, comprising at least one cationic amino acid.

[Claim 5] A stable pharmaceutical formulation according to any one of claims 1-4, comprising 1 - 500 mM histidine and/or citrate buffer, 1-1500 mM of at least one cationic amino acid, 1-200 mg/mL antibody, and 1-400 mM of a carbohydrate.

[Claim 6] The formulation according to claim 4 or 5, whereby the cationic amino acid is arginine.

[Claim 7] The formulation according to claim 5 or 6, whereby the carbohydrate is sucrose or trehalose.

[Claim 8] The formulation according to any one of claims 1-7 further comprising a surfactant.

[Claim 9] The formulation according to any one of claims 1-8 containing the polypeptide and/or antibody in an amount of at least 10 mg/ml.

[Claim 10] The formulation according to any one of claims 1-9 containing the polypeptide and/or antibody in an amount of at least 50 mg/ml.

[Claim 11] The formulation according to any one of claims 1-10 containing the polypeptide and/or antibody in an amount of at least 80 mg/ml.

[Claim 12] The formulation according to any one of claims 1-11 containing the polypeptide and/or antibody in an amount of less than or equal to 240 mg/ml.

[Claim 13] The formulation according to any one of claims 1-12 having a pH in the range from 4.5 to 7.0.

[Claim 14] The formulation according to claim 13 having a pH range from 5.5 to 6.6.

[Claim 15] The formulation according to any one of claims 1-14, whereby the formulation is liquid.

[Claim 16] The formulation according to claim 15 which has not been subjected to lyophilization during preparation of the formulation.

[Claim 17] The formulation according to any one of claims 1-16, wherein the dimerization of the polypeptide and/or antibody molecules is reduced.

[Claim 18] The formulation according to any one of claims 1-17, wherein the dimerization of the polypeptide and/or antibody molecules is inhibited.

[Claim 19] The formulation according to any one of claims 1-18, which is for sub-

cutaneous administration.

[Claim 20] A method for stabilizing a solution containing the antibody, comprising adding at least one cationic amino acid, wherein the antibody is at least one antibody selected from:

(a) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 1 (CDR1 of VH4-M73), CDR2 comprising the sequence of SEQ ID NO: 2 (CDR2 of VH4-M73), and CDR3 comprising the sequence of SEQ ID NO: 3 (CDR3 of VH4-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 10 (CDR1 of VL1), CDR2 comprising the sequence of SEQ ID NO: 11 (CDR2 of VL1), and CDR3 comprising the sequence of SEQ ID NO: 12 (CDR3 of VL1);

(b) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 4 (CDR1 of VH3-M73), CDR2 comprising the sequence of SEQ ID NO: 5 (CDR2 of VH3-M73), and CDR3 comprising the sequence of SEQ ID NO: 6 (CDR3 of VH3-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 13 (CDR1 of VL3), CDR2 comprising the sequence of SEQ ID NO: 14 (CDR2 of VL3), and CDR3 comprising the sequence of SEQ ID NO: 15 (CDR3 of VL3);

(c) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 7 (CDR1 of VH5-M83), CDR2 comprising the sequence of SEQ ID NO: 8 (CDR2 of VH5-M83), and CDR3 comprising the sequence of SEQ ID NO: 9 (CDR3 of VH5-M83), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 16 (CDR1 of VL5), CDR2 comprising the sequence of SEQ ID NO: 17 (CDR2 of VL5), and CDR3 comprising the sequence of SEQ ID NO: 18 (CDR3 of VL5);

(d) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 19 (variable region of VH4-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 22 (variable region of VL1);

(e) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 20 (variable region of VH3-M73) and a light chain variable region comprising the sequence

of SEQ ID NO: 23 (variable region of VL3);
(f) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 (variable region of VH5-M83) and a light chain variable region comprising the sequence of SEQ ID NO: 24 (variable region of VL5);
(g) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 25 (VH4-M73) and a light chain comprising the sequence of SEQ ID NO: 28 (VL1);
(h) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 26 (VH3-M73) and a light chain comprising the sequence of SEQ ID NO: 29 (VL3); and
(i) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 27 (VH5-M83) and a light chain comprising the sequence of SEQ ID NO: 30 (VL5).

[Claim 21] A method for stabilizing an antibody during freeze/thaw cycles of a solution containing the antibody, comprising adding at least one cationic amino acid, wherein the antibody is at least one antibody selected from:
(a) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 1 (CDR1 of VH4-M73), CDR2 comprising the sequence of SEQ ID NO: 2 (CDR2 of VH4-M73), and CDR3 comprising the sequence of SEQ ID NO: 3 (CDR3 of VH4-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 10 (CDR1 of VL1), CDR2 comprising the sequence of SEQ ID NO: 11 (CDR2 of VL1), and CDR3 comprising the sequence of SEQ ID NO: 12 (CDR3 of VL1);
(b) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 4 (CDR1 of VH3-M73), CDR2 comprising the sequence of SEQ ID NO: 5 (CDR2 of VH3-M73), and CDR3 comprising the sequence of SEQ ID NO: 6 (CDR3 of VH3-M73), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 13 (CDR1 of VL3), CDR2 comprising the sequence of SEQ ID NO: 14 (CDR2 of VL3), and CDR3 comprising the sequence of SEQ ID NO: 15 (CDR3 of VL3);
(c) an antibody which comprises a heavy chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 7 (CDR1 of

VH5-M83), CDR2 comprising the sequence of SEQ ID NO: 8 (CDR2 of VH5-M83), and CDR3 comprising the sequence of SEQ ID NO: 9 (CDR3 of VH5-M83), and a light chain variable region that comprises CDR1 comprising the sequence of SEQ ID NO: 16 (CDR1 of VL5), CDR2 comprising the sequence of SEQ ID NO: 17 (CDR2 of VL5), and CDR3 comprising the sequence of SEQ ID NO: 18 (CDR3 of VL5);

(d) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 19 (variable region of VH4-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 22 (variable region of VL1);

(e) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 20 (variable region of VH3-M73) and a light chain variable region comprising the sequence of SEQ ID NO: 23 (variable region of VL3);

(f) an antibody that comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 (variable region of VH5-M83) and a light chain variable region comprising the sequence of SEQ ID NO: 24 (variable region of VL5);

(g) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 25 (VH4-M73) and a light chain comprising the sequence of SEQ ID NO: 28 (VL1);

(h) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 26 (VH3-M73) and a light chain comprising the sequence of SEQ ID NO: 29 (VL3); and

(i) an antibody that comprises a heavy chain comprising the sequence of SEQ ID NO: 27 (VH5-M83) and a light chain comprising the sequence of SEQ ID NO: 30 (VL5).