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(54) Title: Fc-ERYTHROPOIETIN FUSION PROTEIN WITH IMPROVED PHARMACOKINETICS

(57) Abstract: The present invention provides novel highly sialylated Fc-EPO fusion proteins preferably comprising a couple of modifications in the Fc- portion as well as in the EPO portion and having improved pharmacokinetics. Specifically, the Fc-EPO proteins have a prolonged serum half-life and increased in vivo potency. The Fc-EPO fusion proteins synthesized in BHK cells have dramatically prolonged serum half-lives and increased in vivo potency when compared to corresponding Fc-EPO fusion proteins produced in other cell lines, such as, for example, NS/O cells.

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Fc-ERYTHROPOIETIN FUSION PROTEIN WITH IMPROVED PHARMACOKINETICS

FIELD OF THE INVENTION

The present invention provides novel, as a rule, highly sialylated Fc-EPO fusion proteins with improved pharmacokinetics. Specifically, the Fc-EPO proteins have a prolonged serum half-life and increased in vivo potency. The Fc-EPO fusion proteins synthesized in BHK cells have dramatically prolonged serum half-lives and increased in vivo potency when compared to corresponding Fc-EPO fusion proteins produced in other cell lines, such as, for example, NS/0 cells. The present invention relates also to Fc-EPO, wherein a couple of modifications in the Fc- portion as well as in the EPO portion has been carried out in order to obtain respective molecules with further improved properties.

BACKGROUND

Erythropoietin is a glycoprotein hormone necessary for the maturation of erythroid progenitor cells into erythrocytes. It is produced in the kidney and is essential in regulating levels of red blood cells in the circulation. Conditions marked by low levels of tissue oxygen signal increases in production of erythropoietin, which in turn stimulates erythropoiesis. The erythropoietin level in the circulation is strictly regulated to ensure that red blood cells are made only in response to a long-term oxygen deficit. 70% of erythropoietin is cleared by receptor-mediated endocytosis. When erythropoietin binds to its receptor, the complex is endocytosed and degraded, thus limiting the extent of signaling. The remainder of erythropoietin is cleared through kidney filtration into the urine. As a result, erythropoietin has a relatively short serum half-life.

Naturally-occurring human erythropoietin or recombinant erythropoietin produced in mammalian cells contains three N-linked and one O-linked oligosaccharide chains. N-linked glycosylation occurs at asparagine residues located at positions 24, 38 and 83, while O-linked glycosylation occurs at a serine residue located at position 126 (Lai *et al.*, (1986) J. Biol. Chem. 261:3116; Broudy *et al.*, (1988) Arch. Biochem. Biophys. 265:329). The oligosaccharide chains have been shown to be modified with terminal sialic acid residues. N-linked chains typically have up to four sialic acids per chain and O-linked chains have up to two sialic acids. An erythropoietin polypeptide may therefore accommodate up to a total of 14 sialic acids. It has been shown that the carbohydrate is

required for secretion of erythropoietin from cells, for increasing the solubility of erythropoietin, and for the *in vivo* biological activity of erythropoietin (Dube *et al.*, (1988) J. Biol. Chem. 263:17516; DeLorme *et al.*, (1992) Biochemistry 31:9871-9876).

Administration of recombinant human erythropoietin has been effective in treating hematopoietic disorders or deficiencies, such as, for example, different forms of anemia, including those associated with renal failure, HIV infection, blood loss and chronic disease. Erythropoietin is typically administered by intravenous injection. Since erythropoietin has a relatively short serum half-life, frequent intravenous injections are required to maintain a therapeutically effective level of erythropoietin in the circulation. Pharmaceutical compositions containing naturally-occurring or recombinant human erythropoietin are typically administered three times per week at a dose of approximately 25-100 Units/kg. This form of erythropoietin therapy, although quite effective, is very expensive and inconvenient because intravenous administration often necessitates a visit to a doctor or hospital. Currently, a hyperglycosylated recombinant human erythropoietin analogue, novel erythropoiesis stimulating protein (NESP), is available under the trademark Aranesp® (Amgen Inc., Thousand Oaks, California) for treatment of anemia. Aranesp® can be administered less frequently than regular erythropoietin to obtain the same biological response.

An alternative route of administration is subcutaneous injection. This form of administration may be performed by patients at home, and is more compatible with slow-release formulations offering slower absorption from the site of administration, thus causing a sustained release effect. However, significantly lower circulation levels are achieved by subcutaneous injection and, thus, frequent injections are required to achieve desirable therapeutic effect. Furthermore, subcutaneous administration of protein drugs is generally more immunogenic than intravenous administration because the skin, as the major barrier to infection, is an immune organ that is rich in dendritic cells and has sensitive mechanisms for identifying and responding to abrasions and foreign materials. Casadevall *et al.* recently reported that patients receiving erythropoietin subcutaneously developed anti-erythropoietin antibodies (Casadevall *et al.* (2002) N Engl. J. Med. 346(7):469-75).

Accordingly, there is a need for a more efficient erythropoietin therapy that requires less frequent administrations.

SUMMARY OF THE INVENTION

The present invention provides erythropoietin fusion proteins with improved pharmacokinetics compared, in various embodiments, to wild-type or naturally-occurring erythropoietin, to recombinant erythropoietin, or to hyperglycosylated erythropoietin analogue NESP (PCT publication WO 00/24893). Accordingly, it is an object of the present invention to simplify erythropoietin therapy and to reduce the costs associated with treating humans or other mammals with hematopoietic disorders or deficiencies or other indications for erythropoietin administration.

Specifically, the present invention provides a biologically active Fc-erythropoietin (Fc-EPO) fusion protein that has prolonged serum half-life and increased *in vivo* potency.

"Fc-EPO fusion protein," as used herein, refers to a protein comprising a polypeptide having an Fc portion and an erythropoietin portion. "Fc portion," as used herein, encompasses domains derived from the constant region of an immunoglobulin, preferably a human immunoglobulin, including a fragment, analog, variant, mutant or derivative of the constant region. "Erythropoietin portion," as used herein, encompasses wild-type or naturally-occurring erythropoietin from human and other species, recombinant erythropoietin, and erythropoietin-like molecules, including biologically-active erythropoietin fragments, analogs, variants, mutants or derivatives of erythropoietin.

In one aspect, the present invention provides Fc-EPO proteins synthesized in BHK cells. The inventive Fc-EPO fusion proteins synthesized in BHK cells have demonstrated dramatically prolonged serum half-lives and increased *in vivo* potency when compared to corresponding Fc-EPO fusion proteins produced in other cell lines, such as, for example, NS/0, PerC6, or 293 cells. The present invention also provides a population of highly sialylated Fc-EPO fusion proteins suitable for administration to a mammal. The highly sialylated Fc-EPO fusion proteins have longer serum half-lives and increased *in vivo* potency compared, in various embodiments, to wild-type or naturally-occurring erythropoietin, to recombinant erythropoietin, to hyperglycosylated erythropoietin analogue NESP, or to Fc-EPO fusion proteins of the same amino acid sequence synthesized in NS/0, PerC6, or 293 cells. In accordance with the present invention, an Fc-EPO fusion protein can contain amino acid modifications in the Fc portion that generally extend the serum half-life of an Fc fusion protein. For example, such amino acid modifications include mutations substantially decreasing or eliminating Fc receptor

binding or complement fixing activity. In addition, the Fc-EPO fusion protein can also contain amino acid modifications in the erythropoietin portion that reduce EPO receptor-mediated endocytosis or increase the biological activity of erythropoietin. In various embodiments, the present invention combines the benefits provided by an immunoglobulin fusion protein, amino acid modifications of the Fc and erythropoietin portions, and production in BHK cells (*e.g.*, high levels of sialylation). The combined benefits have additive or synergistic effects resulting in an Fc-EPO fusion protein with a surprisingly prolonged serum half-life and an increased *in vivo* potency.

Accordingly, the present invention in one aspect relates to a BHK cell containing a nucleic acid sequence encoding an Fc-EPO fusion protein. In one embodiment, the BHK cell of the present invention is adapted for growth in a protein-free medium. In another embodiment, the BHK cell is adapted for growth in suspension. In yet another embodiment, the BHK cell is adapted for growth in a protein-free medium and in suspension. It has been found that the Fc-EPO fusion proteins produced from BHK cells grown in a protein-free medium exhibited surprisingly increased and more homogeneous sialylation compared to Fc-EPO fusion proteins produced from BHK cells grown in other media. In a preferred embodiment, the nucleic acid is stably maintained in the BHK cell. "Stably maintained nucleic acid," as used herein, refers to any nucleic acid whose rate of loss from a mother cell to a daughter cell is less than three percent in the absence of selective pressure, such as an antibiotic-based selection, to maintain the nucleic acid. Thus, when cells stably maintaining a nucleic acid divide, at least 97 percent (and, more preferably, more than 98, more than 99, or more than 99.5 percent) of the resulting cells contain the nucleic acid. When the resulting cells containing the nucleic acid divide, at least 97 percent of the cells resulting from that (second) division will contain the nucleic acid. Furthermore, the number of copies per cell of the nucleic acid is not substantially reduced by repeated cell division. In a preferred embodiment, the stably maintained nucleic acid sequence is integrated in a chromosome of a BHK cell.

The nucleic acid sequence can encode the Fc-EPO fusion protein in any of various configurations. In a preferred embodiment, the nucleic acid sequence encodes an Fc-EPO fusion protein that includes an Fc portion towards the N-terminus of the Fc-EPO fusion protein and an erythropoietin portion towards the C-terminus of the Fc-EPO fusion protein. The Fc portion generally encompasses regions derived from the constant region of an immunoglobulin, including a fragment, analog, variant, mutant or derivative of the

constant region. In preferred embodiments, the Fc portion is derived from a human immunoglobulin heavy chain, for example, IgG1, IgG2, IgG3, IgG4, or other classes. In some embodiments, the Fc-EPO fusion protein does not include a variable region of an immunoglobulin. In one embodiment, the Fc portion includes a CH2 domain. In another embodiment, the Fc portion includes CH2 and CH3 domains.

In a preferred embodiment, the Fc portion contains a mutation that reduces affinity for an Fc receptor or reduces Fc effector function. For example, the Fc portion can contain a mutation that eliminates the glycosylation site within the Fc portion of an IgG heavy chain. In some embodiments, the Fc portion contains mutations, deletions, or insertions at an amino acid position corresponding to Leu234, Leu235, Gly236, Gly237, Asn297, or Pro331 of IgG1 (amino acids are numbered according to EU nomenclature). In a preferred embodiment, the Fc portion contains a mutation at an amino acid position corresponding to Asn297 of IgG1. In alternative embodiments, the Fc portion contains mutations, deletions, or insertions at an amino acid position corresponding to Leu281, Leu282, Gly283, Gly284, Asn344, or Pro378 of IgG1.

In some embodiments, the Fc portion contains a CH2 domain derived from a human IgG2 or IgG4 heavy chain. Preferably, the CH2 domain contains a mutation that eliminates the glycosylation site within the CH2 domain. In one embodiment, the mutation alters the asparagine within the Gln-Phe-Asn-Ser amino acid sequence within the CH2 domain of the IgG2 or IgG4 heavy chain. Preferably, the mutation changes the asparagine to a glutamine. Alternatively, the mutation alters both the phenylalanine and the asparagine within the Gln-Phe-Asn-Ser amino acid sequence. In one embodiment, the Gln-Phe-Asn-Ser amino acid sequence is replaced with a Gln-Ala-Gln-Ser amino acid sequence.

The asparagine within the Gln-Phe-Asn-Ser amino acid sequence corresponds to Asn297 of IgG1. It has been found that mutation of the asparagine within the Gln-Phe-Asn-Ser amino acid sequence of IgG2 or IgG4 (*i.e.*, corresponding to Asn297 of IgG1) also surprisingly reduces the binding of the Fc-EPO fusion protein for the EPO receptor. Without wishing to be bound by theory, the mutation of the asparagine within the Gln-Phe-Asn-Ser amino acid sequence of IgG2 or IgG4 (*i.e.*, corresponding to Asn297 of IgG1) may induce an overall conformational change in the Fc-EPO fusion protein, leading to dramatically improved pharmacokinetic properties.

In another embodiment, the Fc portion includes a CH2 domain and at least a portion of a hinge region. The hinge region can be derived from an immunoglobulin heavy chain, *e.g.*, IgG1, IgG2, IgG3, IgG4, or other classes. Preferably, the hinge region is derived from human IgG1, IgG2, IgG3, IgG4, or other suitable classes. More preferably the hinge region is derived from a human IgG1 heavy chain. In one embodiment the cysteine in the Pro-Lys-Ser-Cys-Asp-Lys amino acid sequence of the IgG1 hinge region is altered. In a preferred embodiment the Pro-Lys-Ser-Cys-Asp-Lys amino acid sequence is replaced with a Pro-Lys-Ser-Ser-Asp-Lys amino acid sequence. In one embodiment, the Fc portion includes a CH2 domain derived from a first antibody isotype and a hinge region derived from a second antibody isotype. In a specific embodiment, the CH2 domain is derived from a human IgG2 or IgG4 heavy chain, while the hinge region is derived from an altered human IgG1 heavy chain.

In a preferred embodiment, the Fc portion is derived from an IgG sequence in which the Leu-Ser-Leu-Ser amino acid sequence near the C-terminus of the constant region is altered to eliminate potential junctional T-cell epitopes. For example, in one embodiment, the Leu-Ser-Leu-Ser amino acid sequence is replaced with an Ala-Thr-Ala-Thr amino acid sequence. In another embodiment, the Fc portion is derived from an IgG sequence in which the C-terminal lysine residue is replaced. Preferably, the C-terminal lysine of an IgG sequence is replaced with a non-lysine amino acid, such as alanine, to further increase the serum half-life of the Fc fusion protein.

In accordance with the present invention, the Fc portion can contain one or more mutations described herein. The combinations of mutations in the Fc portion generally have additive or synergistic effects on the prolonged serum half-life and increased *in vivo* potency of the Fc-EPO fusion protein. Thus, in one exemplary embodiment, the Fc portion can contain (i) a region derived from an IgG sequence in which the Lys-Ser-Lys-Ser amino acid sequence is replaced with an Ala-Thr-Ala-Thr amino acid sequence; (ii) a C-terminal alanine residue instead of lysine; (iii) a CH2 domain and a hinge region that are derived from different antibody isotypes, for example, an IgG2 CH2 domain and an altered IgG1 hinge region; (iv) a mutation that eliminates the glycosylation site within the IgG2-derived CH2 domain, for example, a Gln-Ala-Gln-Ser amino acid sequence instead of the Gln-Phe-Asn-Ser amino acid sequence within the IgG2-derived CH2 domain.

The erythropoietin portion of the Fc-EPO fusion protein can be a full length wild-type or naturally-occurring erythropoietin, a recombinant erythropoietin, or an

erythropoietin-like molecule, such as a biologically-active erythropoietin fragment, analog, variant, mutant or derivative of erythropoietin. Preferably, the erythropoietin portion is derived from a human erythropoietin. In some embodiments, the erythropoietin portion can contain amino acid modifications that reduce binding affinity for EPO receptor or increase the biological activity of erythropoietin. In some embodiments, the erythropoietin portion contains at least one of the following mutations: Arg131→ Glu and Arg139→ Glu (amino acid numbering based on mature human erythropoietin sequence). In other embodiments, the erythropoietin portion contains at least one of the following mutations: His_{32}→ Gly, Ser_{34}→ Arg, and Pro_{90}→ Ala. In yet another embodiment, the erythropoietin portion has a pattern of disulfide bonding distinct from human erythropoietin. For example, the erythropoietin portion can contain one or more of the following amino acid substitutions: a non-cysteine residue at position 29, a non-cysteine residue at position 33, a cysteine residue at position 88, and a cysteine residue at position 139. In one embodiment, the erythropoietin portion contains cysteine residues at positions 7, 29, 88, and 161. In another embodiment, the erythropoietin portion in addition contains one or more of the following substitutions His_{32}→ Gly, Cys_{33}→ Pro, and Pro_{90}→ Ala. In accordance with the present invention, the erythropoietin portion can contain any combination of the mutations described herein.

In some embodiments, the Fc-EPO fusion protein includes a linker between the Fc portion and the erythropoietin portion. If included, the linker generally contains between 1 and 25 amino acids and preferably has no protease cleavage site. The linker can contain an N-linked or an O-linked glycosylation site to block proteolysis. For example, in one embodiment, the linker contains an Asn-Ala-Thr amino acid sequence.

The present invention also relates to a method of producing an Fc-EPO fusion protein. The method includes maintaining BHK cells containing a nucleic acid sequence encoding an Fc-EPO fusion protein under conditions suitable for expression of the encoded Fc-EPO fusion protein, and recovering the expressed Fc-EPO fusion protein. In one embodiment, the BHK cells are cultured in a protein-free medium. In another embodiment, the BHK cells are cultured in suspension. In yet another embodiment, the BHK cells are cultured in a protein-free medium and in suspension. In some embodiments, the nucleic acid is stably maintained in the BHK cells. Generally, the Fc-EPO fusion protein produced in the BHK cells has a longer serum half-life than a

corresponding Fc-EPO fusion protein produced in other cell lines, such as, for example, NS/0, PerC6, or 293 cells.

The present invention provides a pharmaceutical composition containing the Fc-EPO fusion protein produced in BHK cells. In a preferred embodiment, the Fc-EPO fusion protein used in the pharmaceutical composition has not been treated to remove sialic acid residues. The pharmaceutical composition also includes a pharmaceutically acceptable carrier. The present invention also provides a method of treating a mammal by administering the pharmaceutical composition to the mammal. In some embodiments, the treated mammal has a hematopoietic disorder or deficiency. Because the Fc-EPO fusion proteins of the present invention have increased *in vivo* potency and prolonged serum half-life, pharmaceutical compositions containing the Fc-EPO fusion proteins generally require less frequent administration compared to pharmaceutical compositions containing naturally-occurring or recombinant erythropoietin or corresponding Fc-EPO fusion proteins produced in other cells. In a preferred embodiment, the pharmaceutical composition is administered fewer than three times per week (*e.g.*, twice weekly, weekly, or not more than once every ten days, such as once every two weeks, once per month or once every two months).

In another aspect, the present invention provides a method of selecting a BHK cell that stably maintains a nucleic acid encoding a fusion protein including an Fc portion and an erythropoietin portion. The method includes introducing into a BHK cell a nucleic acid sequence encoding hygromycin B and a nucleic acid sequence encoding the fusion protein; and culturing the BHK cell in the presence of hygromycin B. In one embodiment, the nucleic acid sequence encoding hygromycin B and the nucleic acid sequence encoding the fusion protein are present in a single nucleic acid. In another embodiment, the nucleic acid sequence encoding hygromycin B and the nucleic acid sequence encoding the fusion protein are present in two separate nucleic acids.

In another aspect, the present invention provides a population of purified Fc-EPO fusion proteins suitable for administration to a mammal. In a preferred embodiment, the Fc-EPO fusion proteins include an Fc portion toward the N-terminus of the Fc-EPO fusion proteins and an erythropoietin portion towards the C-terminus of the Fc-EPO fusion proteins. In a more preferred embodiment, the population of purified Fc-EPO fusion proteins is highly sialylated, *i.e.*, having an average of 11-28 sialic acid residues per purified Fc-EPO fusion protein. Preferred highly sialylated populations of Fc-EPO

fusion proteins have an average of 13-28, 15-28, 17-28, 19-28, or 21-28 sialic acid residues per purified Fc-EPO fusion protein. For example, one preferred highly sialylated population of Fc-EPO fusion proteins has an average of 20 to 22 sialic acid residues per purified Fc-EPO fusion protein. In a preferred embodiment, the purified Fc-EPO fusion proteins are synthesized in a BHK cell. In one embodiment, the BHK cell is adapted for growth in suspension. In another embodiment, the BHK cell is adapted for growth in a protein-free medium. In yet another embodiment, the BHK cell is adapted for growth in a protein-free medium and in suspension. The highly sialylated population of purified Fc-EPO fusion proteins provided by the present invention has a longer serum half-life compared to a population of corresponding Fc-EPO fusion proteins produced in cells such as, for example, NS/0, PerC6, or 293 cells. In accordance with the present invention, the Fc portion and the erythropoietin portion of the purified Fc-EPO fusion proteins can contain one or more mutations or modifications as described herein, providing a prolonged serum half-life and an increased *in vivo* potency with effects that are additive or synergistic with enhanced sialylation.

The present invention also provides a pharmaceutical composition containing the highly sialylated population of purified Fc-EPO fusion proteins as described herein. A preferred pharmaceutical composition further includes a pharmaceutically acceptable carrier. The present invention further provides a method of treating a mammal including administering to the mammal the pharmaceutical composition containing the highly sialylated population of purified Fc-EPO fusion proteins. In a preferred embodiment, the pharmaceutical composition is administered fewer than three times per week (*e.g.*, twice weekly, weekly, or not more than once every ten days, such as once every two weeks, once per month or once every two months).

- In summary, the invention relates to the following issues:
  A purified dimeric fusion protein, essentially consisting of a dimeric Fc portion of a human IgG molecule comprising a hinge region, a CH2 and a CH3 domain, and human erythropoietin (EPO), wherein each chain of the dimeric Fc portion is linked via its C-terminus directly or via a linker peptide to the N-terminus of an EPO molecule, said fusion protein has the following properties:(i) the molecule is highly sialylated by comprising 15-28 sialic acid residues; (ii) the CH2 domain derives from human IgG2 and is modified by replacing the amino acid residues Phe and Asn within the Gln-Phe-Asn-Ser sequence track of the CH2 domain with Ala and Asn,

thus forming the sequence Gln-Ala-Gln-Ser within the CH2 domain, and(iii) the Leu-Ser-Leu-Ser amino acid sequence track near the C-terminus of the CH3 domain is replaced with Ala-Thr-Ala-Thr.

- A respective dimeric Fc-EPO fusion protein, wherein, additionally, the C-terminal Lys residue of the CH3 domain is replaced with Ala.
- A respective dimeric Fc-EPO fusion protein, wherein the hinge region derives from human IgG1.
- A respective dimeric Fc-EPO fusion protein, wherein said IgG1 hinge region is modified by replacing the amino acid residue Cys within the Pro-Lys-Ser-Cys-Asp-Lys sequence track of the hinge region with a Ser residue, thus forming the sequence Pro-Lys-Ser-Ser-Asp-Lys within the hinge region.
- A respective dimeric Fc-EPO fusion protein, wherein the erythropoietin portion comprises at least one of the following amino acid substitutions:
  (i) a non-cysteine residue at position 29 of the EPO molecule,
  (ii) a non-cysteine residue at position 33 of the EPO molecule,
  (iii) a cysteine residue at position 88 of the EPO molecule, and
  (iv) a cysteine residue at position 139 of the EPO molecule.
- A respective dimeric Fc-EPO fusion protein, wherein a non-Cys amino acid residue is at position 33 of the EPO molecule instead of the original Cys residue, and a Cys residue is at position 88 of the EPO molecule instead of the original Trp residue, thus enabling the EPO portion within the fusion protein to form a Cys_{29} – Cys_{88} disulfide bond.
- A respective dimeric Fc-EPO fusion protein, wherein the non-Cys amino acid residue at position 33 is Pro.
- A respective dimeric Fc-EPO fusion protein, wherein the EPO portion comprises one or more mutations selected from the group:
  (i) $Arg_{131} \rightarrow Glu_{131}$
  (ii) $Arg_{139} \rightarrow Glu_{139}$
  (iii) $His_{32} \rightarrow Gly_{32}$
  (iv) $Ser_{34} \rightarrow Arg_{34}$
  (v) $Pro_{90} \rightarrow Ala_{90}$.
- A respective dimeric Fc-EPO fusion protein, wherein the linker peptide comprises a glycosylation site.

- A respective dimeric Fc-EPO fusion protein, wherein the glycosylation site comprises an Asn-Ala-Thr amino acid sequence.
- A respective dimeric Fc-EPO fusion protein, comprising additionally a CH1 domain.
- A respective Fc-EPO fusion protein, wherein the complete IgG molecule, including CH2, CH3 and optionally CH1 derives from IgG2 and the hinge region derives from IgG1.
- A respective Fc-EPO fusion protein, wherein the complete IgG molecule, including CH2, CH3 and the hinge region, and optionally CH1, derives from IgG1.
- A respective dimeric Fc-EPO fusion protein, wherin the fusion protein has 18-24, preferably 20 – 22 sialic acid residues.
- A dimeric Fc-EPO fusion protein comprising the sequence:
EPKSSDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWY
VDGVEVHNAKTKPREEQAQSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTK
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSATATPGAAPPRLICDSRVLERYLL
EAKEAENITTGCAEHCSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSEAVLRGQA
LLVNSSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPDAASAAPLRTITADTFRKL
FRVYSNFLRGKLKLYTGEACRTGDR (SEQ ID NO:14).
- A dimeric Fc-EPO fusion protein comprising the sequence:
EPKSSDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWY
VDGVEVHNAKTKPREEQAQSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTK
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSATATPGAAPPRLICDSRVLERYLL
EAKEAENITTGCAEGPSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSEAVLRGQA
LLVNSSQPCEALQLHVDKAVSGLRSLTTLLRALGAQKEAISPPDAASAAPLRTITADTFRKL
FRVYSNFLRGKLKLYTGEACRTGDR (SEQ ID NO:15).
- A DNA molecule encoding a fusion protein as specified above.
- A pharmaceutical composition suitable for the treatment of hematopoietic disorders of deficiencies in a mammal comprising in an effective amount an Fc-EPO fusion protein as specified above or in the claims, optionally together with a pharmaceutically acceptable carrier, diluent or excipient.
- A population of purified highly sialylated Fc-EPO fusion proteins suitable for administration to a mammal, the Fc-EPO fusion proteins comprising an Fc portion towards the N-terminus of the Fc-EPO fusion proteins and an erythropoietin portion

towards the C-terminus of the Fc-EPO fusion proteins, said population of fusion proteins having an average of 15-28 sialic acid residues per purified Fc-EPO fusion protein and being obtainable by introducing a DNA molecule encoding a respective Fc-EPO fusion protein into a BHK cell, and expressing, isolating and purifying the population of corresponding Fc-EOP fusion proteins, wherein said population has a longer serum half-life compared to a population of corresponding Fc-EPO fusion proteins synthesized in NS/0, PerC6, or 293 cells.

- A corresponding population of purified Fc-EPO fusion proteins, wherein said population of fusion proteins has an average of 20-22 sialic acid residues per purified Fc-EPO fusion protein.
- A corresponding population of purified Fc-EPO fusion proteins, wherein the BHK cell is adapted for growth in a protein-free medium or in suspension.
- A method of producing a population of highly sialylated purified recombinant Fc-EPO fusion proteins comprising an Fc portion towards the N-terminus of the Fc-EPO fusion proteins and an erythropoietin portion towards the C-terminus of the Fc-EPO fusion proteins, said method comprising the steps:
  (i) constructing a DNA molecule encoding a Fc-EPO fusion protein;
  (ii) transforming a BHK cell with said DNA molecule in a protein –free medium or in suspension,
  (iii) expressing the population of Fc-fusion proteins encoded by said DNA molecule,
  (iv) harvesting, isolating and purifying said population of Fc-EPO fusion proteins.
- A corresponding method, wherein said synthesized population of fusion proteins has an average of 15-28, preferably 15 – 25, more preferably 20 – 22 sialic acid residues per purified Fc-EPO fusion protein.
- A method of selecting a BHK cell stably maintaining a nucleic acid sequence encoding an Fc-EPO fusion protein comprising an Fc portion and an erythropoietin portion, the method comprising the steps of: (a) introducing into a BHK cell a nucleic acid sequence encoding hygromycin B and a nucleic acid sequence encoding the Fc-EPO fusion protein; and (b) culturing the BHK cell in the presence of hygromycin B.
- A corresponding method, wherein the nucleic acid sequence encoding hygromycin B and the nucleic acid sequence encoding the Fc-EPO fusion protein are present in a single DNA molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B depict an alignment of the amino acid sequences of constant regions of human IgG1, IgG2 and IgG4.

Figure 2 depicts a pharmacokinetics experiment in mice showing a correlation between Fc-EPO dose and amount of decrease in the Fc-EPO serum concentrations during the alpha phase. In this experiment an undersialylated Fc-EPO variant synthesized in NS/0 cells was used.

Figure 3 depicts potential routes of elimination of Fc-EPO fusion proteins and modifications to the fusion protein that potentially modulate these routes.

Figure 4 depicts exemplary hematocrit responses in mice following administration of Fcg2h(FN>AQ)-EPO.

Figure 5 depicts exemplary hematocrit responses in rats following administration of Fcg2h-EPO, Fcg2h-EPO(NDS), Fcg4h-EPO, and Fcg4h(N>Q)-EPO proteins produced from BHK cells.

Figure 6 depicts exemplary hematocrit responses in mice following administration of Fcg2h-EPO(NDS) produced from BHK cells, Fcg2h-EPO(NDS) produced from NS/0 cells, and NESP (*i.e.*, Aranesp®).

Figure 7 depicts an exemplary nucleic acid sequence encoding a mature Fc-EPO protein.

Figure 8 depicts pharmacokinetic profiles of Fcg2h(N>Q)-EPO produced from BHK cells and Fcg2h(N>Q)-EPO produced from NS/0 cells in mice.

Figure 9 depicts pharmacokinetic profiles of Fcg2h-EPO(NDS) produced from BHK cells and Fcg2h-EPO(NDS) produced from NS/0 cells in mice.

Figure 10 depicts pharmacokinetic profiles of Fcg2h-EPO(NDS) proteins produced in BHK-21 cells, PERC6 cells, and 293 cells in mice.

Figure 11 depicts hematocrit responses in beagle dogs following treatment with Fcg2h(FN→AQ)-EPO proteins synthesized in BHK cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an Fc-EPO fusion protein with improved pharmacokinetics. Specifically, the Fc-EPO protein provided by the present invention has a prolonged serum half-life and increased in vivo potency. In one aspect, the present invention provides an Fc-EPO fusion protein synthesized in BHK cells. The Fc-EPO

fusion proteins synthesized in BHK cells have demonstrated dramatically prolonged serum half-lives and increased in vivo potency when compared to corresponding Fc-EPO fusion proteins produced in other cell lines, such as, for example, NS/0, PerC6, or 293 cells. In another aspect, the present invention provides a population of highly sialylated Fc-EPO fusion proteins. The population of highly sialylated Fc-EPO fusion proteins has a longer serum half-life compared to a population of corresponding Fc-EPO fusion proteins with lower levels of sialylation. In accordance with the present invention, an Fc-EPO fusion protein can contain amino acid modifications in the Fc portion that extend serum half-life of an Fc fusion protein, such as by substantially decreasing or eliminating Fc receptor binding activity, or modifications that reduce complement fixing activity. In addition, the Fc-EPO fusion protein can also contain amino acid modifications in the erythropoietin portion that reduce EPO receptor-mediated endocytosis or increase the biological activity of erythropoietin.

Fc-EPO fusion protein

"Fc-EPO fusion protein" as used herein refers to a protein comprising a polypeptide having at least two portions, namely, an Fc portion and an erythropoietin portion, that are not normally present in the same polypeptide. In preferred embodiments of the present invention, the polypeptides having an Fc portion and an erythropoietin portion form homodimers; accordingly, an Fc-EPO fusion protein is generally a dimeric protein held together by one or more disulfide bonds, each polypeptide chain containing an Fc portion and an erythropoietin portion. However, an Fc-EPO fusion protein of the present invention can have any configuration allowing erythropoietin portions to stably associate with Fc portions while maintaining erythropoietin activity. For example, such configurations include, but are not limited to, a single polypeptide containing two Fc portions and two erythropoietin portions, a single polypeptide containing two Fc portions and one erythropoietin portion, a heterodimeric protein including one polypeptide containing an Fc portion and an erythropoietin portion and another polypeptide containing an Fc portion, and other suitable configurations.

The erythropoietin portion can be directly or indirectly linked to the Fc portion in various configurations. In one embodiment, the erythropoietin portion is directly linked to the Fc portion through a covalent bond. For example, the erythropoietin portion can be fused directly to the Fc portion at either its C-terminus or its N-terminus. In one

embodiment, the C-terminus of the Fc portion is fused to the N-terminus of the erythropoietin portion, *i.e.*, N_{term}-Fc-C_{term}-N_{term}-EPO-C_{term}. In this configuration, the Fc portion is towards the N-terminus of the Fc-EPO fusion protein and the erythropoietin portion is towards the C-terminus. In another embodiment, the C-terminus of erythropoietin is fused to the N-terminus of the Fc portion, *i.e.*, N_{term}-EPO-C_{term}-N_{term}-Fc-C_{term}. In this configuration, the erythropoietin portion is towards the N-terminus of the Fc-EPO fusion protein and the Fc portion is towards the C-terminus.

In other embodiments, the erythropoietin portion is indirectly linked to the Fc portion. For example, the Fc-EPO fusion protein can include a linker (L) between the Fc portion and the erythropoietin portion. Similar to the direct fusion, the erythropoietin portion is preferably fused to the C-terminus of the Fc portion through a linker, *i.e.*, N_{term}-Fc-C_{term}-L-N_{term}-EPO-C_{term}. Thus, the Fc portion is towards the N-terminus of the Fc-EPO fusion protein and separated by a linker from the erythropoietin portion towards the C-terminus. Alternatively, the erythropoietin portion can be fused to the N-terminus of the Fc portion through a linker, *i.e.*, N_{term}-EPO-C_{term}-L-N_{term}-Fc-C_{term}.

Fc portion

As used herein, "Fc portion" encompasses domains derived from the constant region of an immunoglobulin, preferably a human immunoglobulin, including a fragment, analog, variant, mutant or derivative of the constant region. Suitable immunoglobulins include IgG1, IgG2, IgG3, IgG4, and other classes. The constant region of an immunoglobulin is defined as a naturally-occurring or synthetically-produced polypeptide homologous to the immunoglobulin C-terminal region, and can include a CH1 domain, a hinge, a CH2 domain, a CH3 domain, or a CH4 domain, separately or in combination. A sequence alignment of the constant regions of human IgG1, IgG2 and IgG4 is shown in Figures 1A and 1B. According to Paul, (1999) Fundamental Immunology 4th Ed., Lippincott-Raven, CH1 domain includes amino acids 118 – 215; hinge region includes amino acids 216 – 230; CH2 domain includes amino acids 231 – 340; and CH3 domain includes amino acids 341 – 447 (the amino acid positions are based on IgG1 sequence). The hinge region joins the CH1 domain to the CH2 and CH3 domains.

In the present invention, the Fc portion typically includes at least a CH2 domain. For example, the Fc portion can include hinge-CH2-CH3. Alternatively, the Fc portion can include all or a portion of the hinge region, the CH2 domain and/or the CH3 domain.

The constant region of an immunoglobulin is responsible for many important antibody functions including Fc receptor (FcR) binding and complement fixation. There are five major classes of heavy chain constant region, classified as IgA, IgG, IgD, IgE, IgM, each with characteristic effector functions designated by isotype. For example, IgG is separated into four γ subclasses: γ1, γ2, γ3, and γ4, also known as IgG1, IgG2, IgG3, and IgG4, respectively.

IgG molecules interact with multiple classes of cellular receptors including three classes of Fcγ receptors (FcγR) specific for the IgG class of antibody, namely FcγRI, FcγRII, and FcγRIII. The important sequences for the binding of IgG to the FcγR receptors have been reported to be located in the CH2 and CH3 domains. The serum half-life of an antibody is influenced by the ability of that antibody to bind to an Fc receptor (FcR). Similarly, the serum half-life of immunoglobulin fusion proteins is also influenced by the ability to bind to such receptors (Gillies SD *et al.*, (1999) Cancer Res. 59:2159-66). Compared to those of IgG1, CH2 and CH3 domains of IgG2 and IgG4 have biochemically undetectable or reduced binding affinity to Fc receptors. It has been reported that immunoglobulin fusion proteins containing CH2 and CH3 domains of IgG2 or IgG4 had longer serum half-lives compared to the corresponding fusion proteins containing CH2 and CH3 domains of IgG1 (U.S. Patent No. 5,541,087; Lo *et al.*, (1998) Protein Engineering, 11:495-500). Accordingly, preferred CH2 and CH3 domains for the present invention are derived from an antibody isotype with reduced receptor binding affinity and effector functions, such as, for example, IgG2 or IgG4. More preferred CH2 and CH3 domains are derived from IgG2.

The hinge region is normally located C-terminal to the CH1 domain of the heavy chain constant region. In the IgG isotypes, disulfide bonds typically occur within this hinge region, permitting the final tetrameric molecule to form. This region is dominated by prolines, serines and threonines. When included in the present invention, the hinge region is typically at least homologous to the naturally-occurring immunoglobulin region that includes the cysteine residues to form disulfide bonds linking the two Fc moieties. Representative sequences of hinge regions for human and mouse immunoglobulins can be found in Borrebaeck, C. A. K., ed., (1992) ANTIBODY ENGINEERING, A PRACTICAL GUIDE, W. H. Freeman and Co. Suitable hinge regions for the present invention can be derived from IgG1, IgG2, IgG3, IgG4, and other immunoglobulin classes. The IgG1 hinge region has three cysteines, two of which are involved in disulfide bonds between

the two heavy chains of the immunoglobulin. These same cysteines permit efficient and consistent disulfide bonding formation between Fc portions. Therefore, a preferred hinge region of the present invention is derived from IgG1, more preferably from human IgG1. In some embodiments, the first cysteine within the human IgG1 hinge region is mutated to another amino acid, preferably serine. The IgG2 isotype hinge region has four disulfide bonds that tend to promote oligomerization and possibly incorrect disulfide bonding during secretion in recombinant systems. A suitable hinge region can be derived from an IgG2 hinge; the first two cysteines are each preferably mutated to another amino acid. The hinge region of IgG4 is known to form interchain disulfide bonds inefficiently. However, a suitable hinge region for the present invention can be derived from the IgG4 hinge region, preferably containing a mutation that enhances correct formation of disulfide bonds between heavy chain-derived moieties (Angal S, *et al.* (1993) Mol. Immunol., 30:105-8).

In accordance with the present invention, the Fc portion can contain CH2 and/or CH3 domains and a hinge region that are derived from different antibody isotypes, *i.e.*, a hybrid Fc portion. For example, in one embodiment, the Fc portion contains CH2 and/or CH3 domains derived from IgG2 or IgG4 and a mutant hinge region derived from IgG1. Alternatively, a mutant hinge region from another IgG subclass is used in a hybrid Fc portion. For example, a mutant form of the IgG4 hinge that allows efficient disulfide bonding between the two heavy chains can be used. A mutant hinge can also be derived from an IgG2 hinge in which the first two cysteines are each mutated to another amino acid. Such hybrid Fc portions facilitate high-level expression and improve the correct assembly of the Fc-EPO fusion proteins. Assembly of such hybrid Fc portions has been described in U.S. Patent Publication No. 20030044423 (*i.e.*, U.S. Application No. 10/093,958), the disclosure of which is hereby incorporated by reference.

In some embodiments, the Fc portion contains amino acid modifications that generally extend the serum half-life of an Fc fusion protein. Such amino acid modifications include mutations substantially decreasing or eliminating Fc receptor binding or complement fixing activity. For example, the glycosylation site within the Fc portion of an immunoglobulin heavy chain can be removed. In IgG1, the glycosylation site is Asn297. In other immunoglobulin isotypes, the glycosylation site corresponds to Asn297 of IgG1. For example, in IgG2 and IgG4, the glycosylation site is the asparagine within the amino acid sequence Gln-Phe-Asn-Ser. Accordingly, a mutation of Asn297 of

IgG1 removes the glycosylation site in an Fc portion derived from IgG1. In one embodiment, Asn297 is replaced with Gln. Similarly, in IgG2 or IgG4, a mutation of asparagine within the amino acid sequence Gln-Phe-Asn-Ser removes the glycosylation site in an Fc portion derived from IgG2 or IgG4 heavy chain. In one embodiment, the asparagine is replaced with a glutamine. In other embodiments, the phenylalanine within the amino acid sequence Gln-Phe-Asn-Ser is further mutated to eliminate a potential non-self T-cell epitope resulting from asparagine mutation. For example, the amino acid sequence Gln-Phe-Asn-Ser within an IgG2 or IgG4 heavy chain can be replaced with a Gln-Ala-Gln-Ser amino acid sequence.

It has also been observed that alteration of amino acids near the junction of the Fc portion and the non-Fc portion can dramatically increase the serum half-life of the Fc fusion protein (PCT publication WO 01/58957, the disclosure of which is hereby incorporated by reference). Accordingly, the junction region of an Fc-EPO fusion protein of the present invention can contain alterations that, relative to the naturally-occurring sequences of an immunoglobulin heavy chain and erythropoietin, preferably lie within about 10 amino acids of the junction point. These amino acid changes can cause an increase in hydrophobicity by, for example, changing the C-terminal lysine of the Fc portion to a hydrophobic amino acid such as alanine or leucine.

In other embodiments, the Fc portion contains amino acid alterations of the Leu-Ser-Leu-Ser segment near the C-terminus of the Fc portion of an immunoglobulin heavy chain. The amino acid substitutions of the Leu-Ser-Leu-Ser segment eliminate potential junctional T-cell epitopes. In one embodiment, the Leu-Ser-Leu-Ser amino acid sequence near the C-terminus of the Fc portion is replaced with an Ala-Thr-Ala-Thr amino acid sequence. In other embodiments, the amino acids within the Leu-Ser-Leu-Ser segment are replaced with other amino acids such as glycine or proline. Detailed methods of generating amino acid substitutions of the Leu-Ser-Leu-Ser segment near the C-terminus of an IgG1, IgG2, IgG3, IgG4, or other immunoglobulin class molecule have been described in U.S. Patent Publication No. 20030166877 (*i.e.*, U.S. patent application No. 10/112,582), the disclosure of which is hereby incorporated by reference.

Erythropoietin portion

As used herein, "erythropoietin portion" encompasses wild-type or naturally-occurring erythropoietin from human and other species, recombinant erythropoietin, and

erythropoietin-like molecules, including biologically-active erythropoietin fragments, analogs, variants, mutants or derivatives of erythropoietin.

Wild-type or naturally-occurring erythropoietin is a 34 KD glycoprotein hormone that stimulates the growth and development of red blood cells from erythropoietin precursor cells. Wild-type or naturally-occurring erythropoietin is produced in the kidney in response to hypoxia (*e.g.*, red blood cell loss due to anemia) and regulates red blood cell growth and differentiation through interaction with its cognate cellular receptor. Wild-type or naturally-occurring erythropoietin can be isolated and purified from blood (Miyake T., *et al.*, (1977) J. Biol. Chem., 252:5558-5564), or plasma (Goldwasser, E., *et al.*, (1971) Proc. Natl. Acad. Sci. U.S.A., 68:697-698), or urine.

Recombinant or chemically- synthesized erythropoietin can be produced using techniques well known to those of skill in the art. Two forms of recombinant human erythropoietin (rHuEPO) are commercially available: EPOGEN® from Amgen and PROCRIT® from Johnson & Johnson.

As used herein, the biological activity of erythropoietin is defined as the ability to stimulate cell proliferation through interaction with the erythropoietin receptor. The functional assay of erythropoietin can be conducted *in vitro* or *in vivo*. For example, the *in vitro* activity of erythropoietin can be tested in a cell-based assay. Specifically, the erythropoietin activity can be determined based on a TF-1 cell proliferation assay. TF-1 cells express EPO receptors. The proliferation of TF-1 cells, which is determined by the incorporation of tritiated thymidine, is a function of erythropoietin activity (Hammerlling *et al.*, (1996) J. Pharmaceutical and Biomedical Analysis, 14:1455; Kitamura *et al.*, (1989) J. Cellular Physiol., 140:323). The *in vitro* cell-based assay is described in more detail in Example 6. *In vivo* assays are typically conducted in animal models, such as, for example, mice and rats. Examples of *in vivo* assays include, but are not limited to, hematocrit (HCT) assays and reticulocyte assays. HCT assays measure the volume of red blood cells from a blood sample taken from an erythropoietin-treated animal, and are performed by centrifuging blood in capillary tubes and measuring the fraction of the total volume occupied by sedimented red blood cells. The *in vivo* HCT assay is described in more detail in Example 8. Reticulocyte assays measure new red blood cells, also known as reticulocytes, that have recently differentiated from precursor cells and still have remnants of nucleic acids characteristic of the precursor cells. Reticulocytes are measured by sorting red blood cells in a flow cytometer after staining with a nucleic acid-

staining dye such as acridine orange or thiazole orange, and counting the positively-stained reticulocyte fraction.

A biologically-active or functionally-active erythropoietin-like molecule typically shares substantial amino acid sequence similarity or identity (*e.g.*, at least about 55%, about 65%, about 75% identity, typically at least about 80% and most typically about 90-95% identity) with the corresponding sequences of wild-type, or naturally-occurring, erythropoietin and possesses one or more of the functions of wild-type erythropoietin thereof.

Thus, erythropoietin of the present invention is understood to specifically include erythropoietin polypeptides having amino acid sequences analogous to the sequence of wild-type erythropoietin. Such proteins are defined herein as erythropoietin analogs. An "analog" is defined herein to mean an amino acid sequence with sufficient similarity to the amino acid sequence of wild-type erythropoietin to possess the biological activity of the protein. For example, an analog of erythropoietin can contain one or more amino acid changes in the amino acid sequence of wild-type erythropoietin, yet possesses, *e.g.*, the ability to stimulate red blood cell production or maturation. Examples of such amino acid changes include additions, deletions or substitutions of amino acid residues. Erythropoietin of the present invention also encompasses mutant proteins that exhibit greater or lesser biological activity than wild-type erythropoietin, such as described in U.S. Patent No. 5,614,184.

Erythropoietin of the present invention also encompasses biologically active fragments of erythropoietin. Such fragments can include only a part of the full-length amino acid sequence of erythropoietin yet possess biological activity. As used herein, a "biologically active fragment" means a fragment that can exert a biological effect similar to the full length protein. Such fragments can be produced by amino- and carboxy-terminal deletions as well as internal deletions. They also include truncated and hybrid forms of erythropoietin. "Truncated" forms are shorter versions of erythropoietin, for example, with amino terminal, or carboxyl terminal residues removed.

Variations in erythropoietin sequence

The amino acid modifications can be introduced into the erythropoietin portion of the present invention to reduce binding affinity to the EPO receptor; to enhance protein stability; to enhance adoption of a correct, active conformation; to enhance pharmacokinetic properties; to enhance synthesis; or to provide other advantageous

features. For example, EPO receptor-mediated endocytosis is determined by the binding affinity between erythropoietin and EPO receptor. The three-dimensional structure of a complex of human erythropoietin and EPO receptor demonstrates that erythropoietin binding to its receptor is dominated by positive charges on the surface of erythropoietin and negative charges on the EPO receptor. Syed *et al.*, (1998) Nature, 395:511. To reduce the on-rate of binding, mutations can be introduced to replace positively charged amino acids that lie near the erythropoietin–EPO receptor contact surface. For example, in one embodiment, one or both of Arg131 and Arg139 of human erythropoietin can be replaced (the amino acid numbering of EPO sequences being based on mature human EPO). Preferably, Arg131 and Arg139 are replaced with glutamic acid, aspartic acid, or other non-positively charged amino acids. Mutations can be introduced in erythropoietin of other species to replace amino acids corresponding to Arg131 and Arg139 of human erythropoietin. However, to preserve EPO biological activity, those residues which are in the center of the EPO-EPO receptor interaction should be avoided when making alterations in the EPO amino acid sequence.

Alternatively, one can empirically determine those regions or positions which would tolerate amino acid substitutions by alanine scanning mutagenesis (Cunningham *et al.*, (1989) Science, 244, 1081-1085). In this method, selected amino acid residues are individually substituted with a neutral amino acid (*e.g.*, alanine) in order to determine the effects on biological activity.

In one embodiment, the erythropoietin portion contains at least one of the following mutations: His32 → Gly and/or Ser34 → Arg, and Pro90 → Ala. In other embodiments, cysteine substitutions are introduced in erythropoietin to alter patterns of cysteine-cysteine disulfide bonds, resulting in new disulfide bond formation ("NDS mutations"). Naturally-occurring human erythropoietin, which appears to be unique among mammalian erythropoietins, has exactly four cysteines at positions 7, 29, 33, and 161 that form two disulfide bonds. One or more of these cysteine residues of the erythropoietin portion can be altered. To generate an altered disulfide bond, one cysteine residue is mutated to a structurally compatible amino acid such as alanine or serine, and a second amino acid that is nearby in the three-dimensional structure is mutated to cysteine. For example, one of amino acids Gln_{86}, Pro_{87}, Trp_{88}, Glu_{89}, and Leu_{91} can be replaced by Cys. If Trp_{88} is replaced by Cys and Cys_{33} is replaced with another amino acid, the erythropoietin portion will form a Cys_{29}-Cys_{88} disulfide bond that is not found in human

EPO. This bond results in a fusion protein that has greater activity than a fusion protein with a typical Cys_{29}-Cys_{33} disulfide bond. In addition, the Cys_{29}-Cys_{88} fusion protein shows a pronounced increase in activity, compared to the Cys_{29}-Cys_{33} fusion protein, in the presence of other mutations in the erythropoietin portion of the fusion protein. Accordingly, in one embodiment of the present invention, the erythropoietin portion includes at least one of the following amino acid substitutions: a non-cysteine residue at position 29, a non-cysteine residue at position 33, a cysteine residue at position 88, and a cysteine residue at position 139. In one embodiment, the erythropoietin portion contains cysteines at positions 7, 29, 88, and 161. In another embodiment, the erythropoietin portion further contains one or more of the following substitutions: His_{32}→Gly, Cys_{33}→Pro, and Pro_{90}→Ala. In an alternative embodiment, an entirely new disulfide bond is added to the protein by mutating two amino acids to cysteines. To compensate for possible strains in the structure that the Cys mutations might cause, in a preferred Cys - engineered embodiment of this invention, the erythropoietin portion further contains mutations designed to alleviate these potential strains.

Further embodiments relating to cysteine substitutions are described in PCT publication WO 01/36489 (*i.e.*, U.S. Application Serial No. 09/708,506), the disclosure of which is hereby incorporated by reference.

Methods for introducing mutations in erythropoietin are well known in the art. For example, mutations can be introduced by site-directed mutagenesis techniques. It is important to note that a wide variety of site-directed mutagenesis techniques are available and can be used as alternatives to achieve similar results. Other techniques include, but are not limited to, random and semi-random mutagenesis.

Linker

The Fc-EPO fusion proteins according to this invention can include a linker molecule, preferably a peptide linker, between the Fc portion and the erythropoietin portion. A fusion protein with a linker may have improved properties, such as increased biological activity. A linker generally contains between 1 and 25 amino acids (*e.g.*, between 5 and 25 or between 10 and 20 amino acids). The linker can be designed to include no protease cleavage site. Furthermore, the linker can contain an N-linked or an O-linked glycosylation site to sterically inhibit proteolysis. Accordingly, in one embodiment, the linker contains an Asn-Ala-Thr amino acid sequence.

Additional suitable linkers are disclosed in Robinson *et al.*, (1998), Proc. Natl. Acad. Sci. USA; 95, 5929; and U.S. Application Serial No. 09/708,506.

Glycosylation

Naturally-occurring human erythropoietin and recombinant erythropoietin expressed in mammalian cells contain three N-linked and one O-linked oligosaccharide chains. N-linked glycosylation occurs at asparagine residues located at positions 24, 38 and 83, while O-linked glycosylation occurs at a serine residue located at position 126 (Lai *et al.*, (1986) J. Biol. Chem., 261:3116; Broudy *et al.*, (1988) Arch. Biochem. Biophys., 265:329). The oligosaccharide chains have been shown to be modified with terminal sialic acid residues. N-linked chains typically have up to four sialic acids per chain and O-linked chains have up to two sialic acids. An erythropoietin polypeptide can therefore accommodate up to a total of 14 sialic acids.

Sialic acid is the terminal sugar on N-linked or O-linked oligosaccharides. The extent of sialylation is variable from site to site, protein to protein, and can depend on cell culture conditions, cell types, and particular cell clones that are used. It has been found that the Fc-EPO fusion protein of the present invention synthesized in BHK cells is highly sialylated. It has also been found that the extent of sialylation of Fc-EPO fusion protein can be further enhanced by adapting the BHK cells for growth in protein-free media, in suspension, or in protein-free media and in suspension. Certain other commonly used cell lines, such as NS/0, PerC6, or 293 cells fail to produce highly sialylated Fc-EPO fusion protein under standard culture conditions. The extent of sialylation of the Fc-EPO fusion protein produced from different cell lines can be determined by isoelectric focusing (IEF) gel electrophoresis by virtue of their highly negatively charged sialic acid residues; the details of IEF gel electrophoresis are described in Example 5B. The extent of sialylation of the Fc-EPO fusion protein produced in different cell lines can also be qualitatively confirmed by lectin-binding studies using methods familiar to those skilled in the art. An example of a lectin-binding assay is described in Example 5B.

Typically, a population of highly sialylated purified Fc-EPO fusion proteins of the present invention has an average of 11-28 sialic acid residues per purified Fc-EPO fusion protein. Preferred highly sialylated populations of Fc-EPO fusion proteins have an average of 13-28, 15-28, 17-28, 19-28, or 21-28 sialic acid residues per purified Fc-EPO fusion protein. For example, one preferred highly sialylated population of Fc-EPO fusion

proteins has an average of 20 to 22 sialic acid residues per purified Fc-EPO fusion protein. Another preferred population of Fc-EPO fusion proteins has an average of 23-28 sialic acid residues per purified Fc-EPO fusion protein.

Pharmacokinetics of the sialylated Fc-EPO fusion protein

One of the most important factors determining the *in vivo* biological activity of erythropoiesis-stimulating agents is the length of time that the serum concentration of the protein remains above the threshold necessary for erythropoiesis, which is determined by the pharmacokinetics of the erythropoiesis-stimulating agents. The pharmacokinetic profile of the highly sialylated Fc-EPO fusion protein is distinct from that of naturally-occurring or recombinant erythropoietin. The major difference is that the highly sialylated Fc-EPO fusion protein has much longer serum half-life and slower clearance leading to increased *in vivo* biological potency. Without wishing to be bound by theory, sialic acid residues are believed to increase the negative charges on an erythropoietin molecule resulting in decreased on-rate for negatively-charged EPO receptor binding and decreased EPO receptor mediated endocytosis, lengthening the serum half-life. Furthermore, sialic acids also prevent erythropoietin proteins from being endocytosed by the asialoglycoprotein receptors that bind glycoproteins with exposed galactose residues.

In general, most pharmacokinetic profiles of a therapeutic molecule such as erythropoietin show an initial drop in serum concentration (an alpha phase), followed by a more gradual decline (a beta phase) following administration.

Factors influencing the alpha phase

According to small-molecule pharmacokinetic theory, the alpha phase defines a volume of distribution that describes how a molecule partitions into compartments outside the blood. The drop observed in the alpha phase varies widely for different Fc-EPO fusion proteins synthesized in different cell lines. In theory, the difference could be due to variation in the volume of distribution, or due to variations in inter-compartment trafficking. However, it has been observed that there is a correlation between the extent of sialylation and the pharmacokinetic behavior of the Fc-EPO proteins in mice. For example, the Fc-EPO fusion proteins synthesized in BHK cells are highly sialylated and show the best pharmacokinetic profile. The Fc-EPO fusion proteins synthesized in NS/0 cells are somewhat sialylated and have an intermediate pharmacokinetic profile. The Fc-EPO fusion proteins synthesized in 293 and PerC6 cells have little or no sialylation and have a poor pharmacokinetic profile characterized by about a 100-fold drop in serum

concentration in the first 30 minutes. Therefore, a key factor that influences the alpha phase of a particular Fc-EPO fusion protein is the distribution of glycosylation species and the level of sialylation. The Fc-EPO fusion proteins that are undersialylated disappear rapidly.

In addition, as shown in Figure 2, the extent of the drop in the Fc-EPO serum concentrations during the alpha phase varies according to the dose, indicating that this behavior is saturable and most likely receptor-mediated. It is possible that the receptor mediating the alpha phase drop is neither EPO receptor nor Fc receptor, but another receptor such as the asialoglycoprotein receptor. Aranesp® has reduced binding affinity to the EPO receptors compared to normal human erythropoietin because Aranesp® has increased negative charges as a result of additional N-linked glycosylation sites. However, Aranesp® and normal human erythropoietin show similar drops during alpha phases. In addition, since generally the number of the EPO receptors on the cell surface of an erythroid progenitor cell is only approximately 200, these receptors would be completely saturated at much lower doses of erythropoietin than those used in Figure 2. Fc receptors are perhaps unlikely to mediate the dramatic drop in the alpha phase because Fc-EPO fusion proteins with a mutation eliminating the glycosylation site, *e.g.*, a mutation of amino acid corresponding to Asn297 of IgG1, can still show a steep drop in the alpha phase. In addition, although IgG2 CH2 regions, when not aggregated, generally do not bind to Fc receptors, the Fc-EPO proteins containing IgG2 CH2 regions still show a significant drop during alpha phase.

Without wishing to be bound by theory, the drop of the serum concentration of an Fc-EPO fusion protein during alpha phase may be mediated by asialoglycoprotein-receptors via asialoglycoprotein-receptor-mediated endocytosis. Undersialylated Fc-EPO fusion proteins contain exposed galactose residues that can be bound by the asialoglycoprotein receptor resulting in asialoglycoprotein-receptor-mediated endocytosis. As a result, undersialylated Fc-EPO fusion proteins can disappear rapidly.

Factors influencing the beta phase

The drop of the serum concentrations of the Fc-EPO fusion proteins in the beta phase is less steep compared to the drop in the alpha phase. For example, in mice, between 8 and 24 hours following administration, a 2- to 3-fold drop in the serum concentrations of the Fc-EPO fusion proteins is observed. The difference in the drop during the beta phase is also less drastic between different Fc-EPO proteins synthesized in

different cell lines. However, like in the alpha phase, the extent of sialylation correlates with the pharmacokinetic behavior in the beta phase. For example, the Fc-EPO fusion proteins synthesized in BHK cells have a significantly improved beta phase compared to otherwise identical Fc-EPO proteins synthesized in NS/0 cells. EPO receptor-mediated endocytosis appears to be at least partly responsible for the drop in the serum concentration of the Fc-EPO fusion proteins during beta phase. Aranesp®, which has reduced binding affinity for EPO receptors compared to normal human erythropoietin, has a significantly improved beta phase compared to normal human erythropoietin, despite similar alpha phase profiles.

The Fc-EPO fusion proteins of the invention generally exhibit an improved beta phase compared to naturally-occurring or recombinant erythropoietin, indicating that the addition of the Fc portion significantly slows down the decline of the serum concentration during the beta phase. It has also been observed that certain amino acid modifications in the Fc portion or in the erythropoietin portion can significantly improve the beta phase. For example, mutations eliminating the glycosylation site in the Fc portion improve the beta phase of Fc-EPO fusion proteins. Mutations increasing the stability of the erythropoietin portion, *e.g.*, mutations engineering disulfide bonds (for example, NDS mutations) in the erythropoietin portion, significantly improve the beta phase of the Fc-EPO fusion protein. Generally, an improved beta phase extends the terminal serum half-life of an Fc-EPO fusion protein.

Routes of elimination of Fc-EPO fusion proteins

There are several possible routes of elimination of an erythropoietin protein molecule from the body. A wild-type or naturally-occurring erythropoietin protein molecule can be eliminated from the body by kidney filtration and receptor-mediated endocytosis. Endocytosed erythropoietin is efficiently degraded. As depicted in Figure 3, the addition of an Fc portion to the erythropoietin portion is expected to essentially abolish the excretion of the Fc-EPO fusion protein through the kidney. As a result, receptor-mediated endocytosis is the major route of elimination of an Fc-EPO fusion protein. Furthermore, the addition of an Fc portion to the erythropoietin portion is also expected to reduce degradation after internalization, because the FcRn endosomal receptors are expected to recycle the fusion protein back out of the cell.

In principle, at least three types of receptors can mediate the clearance of the Fc-EPO fusion protein, namely, Fc-receptor, EPO receptor, and asialoglycoprotein receptor.

Clearance of the Fc-EPO fusion protein through the Fc receptor should be significantly reduced by use of an IgG2-derived CH2 domain instead of an IgG1-derived CH2 in the Fc portion. IgG2-derived CH2 domains have about a 100-fold lower affinity for FcγRI, which has the highest affinity for IgGs, compared to IgG1-derived CH2 domains. The interaction between the IgG2-derived CH2 and FcγRI is undetectable in most binding assays. However, the residual FcγR-binding activity of the IgG2-derived CH2 domain may still play a role in clearance of Fc-EPO fusion protein because the asparagine mutation eliminating the glycosylation site in the CH2 domain further reduces Fc-receptor binding and improves the pharmacokinetics of the Fc-EPO fusion protein.

The NDS mutations have the effect of stabilizing the erythropoietin structure and, as a result, are expected to reduce degradation of the Fc-EPO fusion protein after internalization. The Fc-EPO fusion proteins containing the NDS mutations have improved pharmacokinetic properties and increased serum half-life.

Sialylation increases the negative charges of Fc-EPO fusion proteins, reducing the binding affinity of the Fc-EPO fusion protein for the EPO receptor. Sialylation also reduces the number of exposed galactose residues on the Fc-EPO fusion protein, reducing binding affinity of the Fc-EPO fusion proteins for the asialoglycoprotein receptors. Accordingly, as depicted in Figure 3, sialylation reduces both EPO receptor-mediated endocytosis and asialoglycoprotein receptor-mediated endocytosis. Highly sialylated Fc-EPO fusion proteins therefore have dramatically slowed clearance rates resulting in significantly increased serum half-lives.

The addition of an Fc portion, the alterations of Fc and erythropoietin portions, and sialylation each reduce the clearance of Fc-EPO fusion proteins. The combined effects on clearance and serum half-life are additive or multiplicative.

In vitro activity and in vivo potency of the Fc-EPO fusion protein

The *in vitro* activity of Fc-EPO proteins can be tested in a cell-based assay. Specifically, the interaction between Fc-EPO and EPO receptor can be determined based on the TF-1 cell proliferation assay. The TF-1 cells express EPO receptors, therefore, the proliferation of TF-1 cells, which is determined by the incorporation of tritiated thymidine, is a function of erythropoietin activity (Hammerlling *et al.*, (1996) J. Pharmaceutical and Biomedical Analysis, 14:1455; Kitamura *et al.*, (1989) J. Cellular Physiol., 140:323). In the present invention, the proliferation of TF-1 cells is a function

of interaction between the erythropoietin portion and EPO receptors. Specifically, if an erythropoietin portion of an Fc-EPO fusion protein has a reduced on-rate for the EPO receptor, the Fc-EPO protein generally has a reduced activity in a cell-based assay (marked by an increased ED50 value).

Data from cell-based assays, which are relatively easy to obtain, generally correlate with pharmacokinetics and *in vivo* potency of the Fc-EPO protein. Reduced *in vitro* activity, indicating a reduced on-rate for the EPO receptor, generally correlates with improved pharmacokinetic properties and enhanced *in vivo* potency. On the contrary, increased *in vitro* activity (marked by a decreased ED50 value), indicating an enhanced on-rate for the EPO receptor, generally correlates with poor pharmacokinetic properties and reduced *in vivo* potency.

The *in vivo* biological activities of Fc-EPO fusion proteins can be measured by assays conducted in animal models, such as, for example, mice and rats. Examples of *in vivo* assays include, but are not limited to, hematocrit (HCT) assays and reticulocyte assays. HCT assays measure the volume of blood occupied by red blood cells (RBCs), and are performed simply by centrifuging blood in capillary tubes and measuring the fraction of the total volume occupied by sedimented RBCs. Reticulocytes are new RBCs that have recently differentiated from precursor cells and characterized by containing remnants of nucleic acids from the precursor cells. Reticulocytes are measured by sorting red blood cells in a flow cytometer after staining with a nucleic acid-staining dye, such as, for example, acridine orange or thiazole orange, and counting the staining fraction. Typically, the hematocrit and reticulocytes are measured twice per week.

Reticulocyte data are, in a sense, a first derivative of the hematocrit data. Reticulocyte counts are a measure of the rate of production of red blood cells, while hematocrits measure the total red blood cells. In a typical experiment, the hematocrits of animals administered with Fc-EPO fusion proteins will increase and then return to baseline. When the hematocrits are high and the administered Fc-EPO proteins have disappeared from the animal's circulation system, the reticulocyte count goes below baseline because erythropoiesis is suppressed.

Reticulocytes normally emerge from the bone marrow 4 days after the precursors committed to RBC fates. However, in the presence of high levels of erythropoietin, reticulocytes will often leave the bone marrow after 1-3 days after administration.

In response to an injection of Fc-EPO proteins, the hematocrit readings increase, remain steady, then return to baseline in an animal. Examples of such hematocrit responses are shown in Figures 4-6. The maximal rate of decrease is about 7% of blood volume per week in mice, which corresponds to the RBC lifetime of about 45 days in a mouse, and about 5% of blood volume per week in rats, which corresponds to the RBC lifetime of about 65 days in a rat. The maximal rate of decrease presumably represents destruction of RBCs in the absence of new synthesis. If biologically-active Fc-EPO proteins remain in the system at a concentration above the threshold for erythropoiesis, the hematocrit level will remain high and not fall, even if the level of biologically-active Fc-EPO is not detectable in pharmacokinetics experiments.

It has been found that the pharmacokinetic properties of an Fc-EPO protein correlates with the *in vivo* potency of the protein. All of the features of the present invention that enhance pharmacokinetics of an Fc-EPO fusion protein, as discussed above, also enhance *in vivo* potency in animal experiments. As shown in Table 1, such features include, for example, addition of the Fc potion, elimination of the glycosylation site in the Fc portion (*e.g.*, N→Q substitution at a position corresponding to Asn297 of IgG1), introduction of the NDS mutations into the erythropoietin portion, and high levels of sialylation by synthesis the Fc-EPO protein in the BHK cells.

Table 1

Factors that influence the pharmacokinetics and biological activity of Fc-EPO proteins

Features	**Effect on *in vitro* potency**	**Effect on pharmacokinetics**	**Effect on *in vivo* activity**
Synthesis in BHK cells (*vs.* NS/0 cells)	Reduction	Enhancement	Enhancement
Addition of Fc	Small enhancement	Enhancement	Enhancement
NDS Mutations	None	Enhancement	Enhancement
N→Q	None	Enhancement	Enhancement
g2h (*vs.* g4h)	Enhancement	Enhancement	Enhancement

It has been found that, per erythropoietin portion, Fcg2h(FN→AQ)-Epo and Fcg2h-EPO(NDS) made from BHK cells show the best pharmacokinetics and most potent *in vivo* biological activities. Fcg2h(FN→AQ)-Epo and Fcg2h-EPO(NDS) each have a

longer serum half life and more potent *in vivo* activity per erythropoietin portion than Aranesp®.

Synthesis of Fc-EPO fusion proteins

The Fc-EPO fusion protein of the present invention can be produced in suitable cells or cell lines such as human or other mammalian cell lines. Suitable cell lines include, but are not limited to, baby hamster kidney (BHK) cells, Chinese hamster ovary (CHO) cells (including dihydrofolate reductase (DHFR)-deficient cells), and COS cells. In a preferred embodiment, BHK cells are used.

To express the Fc-EPO fusion protein in suitable host cells (*e.g.*, BHK cells), nucleic acid sequences encoding the Fc-EPO fusion protein are first introduced into an expression vector using standard recombinant molecular techniques familiar to those ordinarily skilled in the art. The sequence encoding the erythropoietin portion is preferably codon-optimized for high level expression. Codon-optimized human erythropoietin was described in PCT publication WO 01/36489 (*i.e.*, U.S. Application No. 09/708,506), the disclosures of which are hereby incorporated by reference. An exemplary nucleic acid sequence encoding an erythropoietin portion is provided in SEQ ID NO:1:

GCCCCACCACGCCTCATCTGTGACAGCCGAGTGCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGC
CGAGAATATCACGACCGGCTGTGCTGAACACTGCAGCTTGAATGAGAACATCACCGTGCCTGACA
CCAAAGTGAATTTCTATGCCTGGAAGAGGATGGAGGTTGGCCAGCAGGCCGTAGAAGTGTGGCAG
GGCCTGGCCCTGCTGTCGGAAGCTGTCCTGCGGGGCCAGGCCCTGTTGGTCAACTCTTCCCAGCC
GTGGGAGCCCCTGCAACTGCATGTGGATAAAGCCGTGAGTGGCCTTCGCAGCCTCACCACTCTGC
TTCGGGCTCTGGGAGCCCAGAAGGAAGCCATCTCCCCTCCAGATGCGGCCTCAGCTGCTCCCCTC
CGCACAATCACTGCTGACACTTTCCGCAAACTCTTCCGAGTCTACTCCAATTTCCTCCGGGGAAA
GCTGAAGCTGTACACAGGGGAGGCCTGCCGGACAGGGGACAGATGA (SEQ ID NO:1)

Exemplary nucleic acid sequences encoding a preferred Fc portion, for example, an Fc portion including a CH2 domain derived from IgG2 and a hinge region derived from IgG1, was described in U.S. Patent Publication No. 20030044423 (*i.e.*, U.S. Application No. 10/093,958), the disclosure of which is hereby incorporated by reference.

Generally, a nucleic acid sequence encoding an Fc-EPO fusion protein includes a nucleic acid sequence encoding a signal peptide (leader sequence). The leader sequence is cleaved during the secretion process. An exemplary nucleic acid sequence (SEQ ID NO:2) encoding a mature Fc-EPO protein without a leader sequence is shown in Figure 7.

Suitable vectors include those suitable for expression in a mammalian host cell. The vectors can be, for example, plasmids or viruses. The vector will typically contain the following elements: promoter and other "upstream" regulatory elements, origin of replication, ribosome binding site, transcription termination site, polylinker site, and selectable marker that are compatible with use in a mammalian host cell. Vectors may also contain elements that allow propagation and maintenance in prokaryotic host cells as well. Suitable vectors for the present invention includes, but are not limited to, pdCs-Fc-X and vectors derived therefrom, and phC10-Fc-X and vectors derived therefrom.

The vectors encoding Fc-EPO proteins are introduced into host cells by standard cell biology techniques, including transfection and viral techniques. By transfection is meant the transfer of genetic information to a cell using isolated DNA, RNA, or synthetic nucleotide polymer. Suitable transfection methods include, but are not limited to, calcium phosphate-mediated co-precipitation (Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press), lipofection (*e.g.*, Lipofectamine Plus from Life Technologies of Rockville, Maryland), DEAE-dextran-mediated transfection techniques, lysozyme fusion or erythrocyte fusion, scraping, direct uptake, osmotic or sucrose shock, direct microinjection, indirect microinjection such as via erythrocyte-mediated techniques, protoplast fusion, or by subjecting the host cells to electric currents (*e.g.*, electroporation), to name but a few. The above list of transfection methods is not considered to be exhaustive, as other procedures for introducing genetic information into cells will no doubt be developed.

To facilitate selection of the host cells containing the nucleic acid encoding the Fc-EPO fusion protein, the nucleic acid encoding the Fc-EPO fusion protein is typically introduced with a selection marker. The selection marker can be encoded by a nucleic acid sequence present on the same expression vector encoding the Fc-EPO fusion protein. Alternatively, the selection marker can be encoded by a nucleic acid sequence present on a different vector. In the latter case, the two vectors can be co-introduced into the host cells by either cotransfection or co-transduction. Suitable selection markers include, for example, Hygromycin B (Hyg B) and dihydrofolate reductase (DHFR).

Transient expression is useful for small-scale protein production and for rapid analysis of an Fc-EPO fusion protein. The host cells containing the nucleic acid sequence encoding the Fc-EPO fusion protein are maintained under conditions suitable for expression of the encoded Fc-EPO fusion protein. Standard cell culture methods,

conditions and media can be used for maintaining the host cells expressing the Fc-EPO fusion protein.

Stably transfected cells are often preferred for large-scale production, high level expression, and for other purposes. The stably maintained nucleic acid can be present in any of various configurations in the host cell. For example, in one embodiment, the stably maintained nucleic acid sequence is integrated in a chromosome of a host cell. In other embodiments, the stably maintained nucleic acid sequence can be present as an extrachromosomal array, as an artificial chromosome, or in another suitable configuration.

In one embodiment, BHK cells are used to synthesize the Fc-EPO fusion protein. In order to obtain a stably transfected BHK cell, a nucleic acid sequence encoding the fusion protein and a nucleic acid sequence encoding a selection marker are introduced into BHK cells, preferably by electroporation, protoplast fusion or lipofection methods. The nucleic acid sequence encoding the fusion protein and the nucleic acid sequence encoding a selection marker can be present on the same expression vector. Alternatively, the nucleic acid sequence encoding the fusion protein and the nucleic acid sequence encoding a selection marker can be present on separate vectors. The preferred selection marker for establishing a stable BHK cell is Hyg B. Other selection markers, such as DHFR, can also be used. Stably transfected clones are isolated and propagated by their growth in the presence of Hyg B at a suitable concentration (for example, 200, 250, or 300 micrograms/ml), in standard tissue culture medium, such as, for example, MEM+FBS, DMEM/F-12 medium, or VP-SFM available from Life Technologies, and other suitable media. The expression levels of the Fc-EPO fusion protein can be monitored by standard protein-detecting assays, such as, for example, ELISA test, Western Blot, dot blot, or other suitable assays, on samples from supernatants and culture media. High expression clones are selected and propagated in large scale.

Typically, the BHK cell is an adherent cell line and commonly grown in serum-containing media, such as MEM + 10% heat-inactivated fetal bovine serum (FBS). However, the BHK cells can be adapted for growth in suspension and in a serum-free medium, such as, for example, VP-SFM (Invitrogen Corp., cat # 11681-020) or Opti-Pro SFM (Invitrogen Corp., cat # 12309). An exemplary adaptation process is described in Example 3. The BHK cells adapted for growth in a serum-free medium can be further adapted for growth in a protein-free medium, such as, for example, DMEM/F-12

(Invitrogen Corp., cat # 11039-021). One exemplary adaptation procedure is described in Example 3. Preferably, DMEM/F-12 is supplemented with suitable amino acids and other components, such as, for example, Glutamine, protein hydrolysates such as HyPep 4601 (Quest International, cat # 5Z10419) and HyPep 1510 (Quest International, cat # 5X59053), Ethanolamine (Sigma, cat# E0135), and Tropolone (Sigma, cat # T7387). Suitable concentrations of each supplement can be determined empirically by those skilled in the art with routine experimentation.

The Fc-EPO fusion proteins synthesized in BHK cells grown in a protein-free medium are sialylated to a greater extent and exhibit more homogeneous sialylation than the corresponding protein synthesized in cells grown in a serum-containing medium (*e.g.*, MEM + FBS) or a serum-free but not protein-free medium (*e.g.*, VP-SFM). In addition, the Fc-EPO protein thus obtained is substantially non-aggregated, *i.e.*, approximately 98 % of total yield is non-aggregated. The protein yield from BHK cells grown in a protein-free medium is similar to that from BHK cells grown in serum-containing media, *i.e.*, above 10 microgram/milliliter (mcg/ml). Thus, growth in suspension and/or in a protein-free medium offers a number of advantages, including 1) improving pharmacokinetics of the Fc-EPO fusion protein resulted from increased sialylation; and 2) facilitating downstream purification processes because proteins can be purified from cells grown in suspension mode and in a medium devoid of protein.

Purification

Purification of Fc-EPO is done following standard GMP procedures known by persons skilled in the art. The protein is generally purified to homogeneity or near homogeneity. Chromatographic purifications, such as those involving column chromatography, are generally preferred. Generally, a purification scheme for an Fc-EPO fusion protein may include, but is not limited to, an initial protein capture step; a viral inactivation step; one or more polishing steps; a viral removal step; and a protein concentration and/or formulation step. For example, chromatography resin materials that bind to the Fc portion of the fusion protein can be used to capture Fc-EPO proteins. Suitable resin materials include, but are not limited to, resins coupled to Protein A. Polishing steps may be included to remove contaminating components. For example, hydroxyapatite chromatography, Sepharose Q chromatography, size exclusion chromatography, or hydrophobic interaction chromatography may be used to remove

contaminants. One purification method using Protein A-based column chromatography to bind the Fc portion and purify the Fc-EPO fusion protein is described in Example 12, as is an optional method for virus inactivation and removal. The purified proteins are generally concentrated to a desired concentration using ultrafiltration; diafiltered into a suitable formulation buffer; filter sterilized; and dispensed into vials.

Administration

Pharmaceutical compositions and administration routes

The present invention also provides pharmaceutical compositions containing the Fc-EPO protein produced according to the present invention. These pharmaceutical compositions can be used to stimulate red blood cell production and to prevent and to treat anemia. Among the conditions treatable by the present invention include anemia associated with a decline or loss of kidney function (chronic renal failure), anemia associated with myelosuppressive therapy, such as chemotherapeutic or anti-viral drugs (such as AZT), anemia associated with the progression of non-myeloid cancers, anemia associated with viral infections (such as HIV), and anemia of chronic disease. Also treatable are conditions which may lead to anemia in an otherwise healthy individual, such as an anticipated loss of blood during surgery. In general, any condition treatable with rHuEpo can also be treated with the Fc-EPO fusion protein of the invention.

Formulations containing Fc-EPO proteins

Generally, a formulation contains an Fc-EPO protein, a buffer and a surfactant in liquid or in solid form. Solid formulations also include, but are not limited to, freeze-dried, spray-freeze-dried or spray-dried formulations. Liquid formulations are preferably based on water, but can contain other components, such as, for example, ethanol, propanol, propanediol or glycerol, to name but a few.

Fc-EPO proteins are formulated in aqueous solutions following standard GMP procedures known to persons skilled in the art. Generally, a formulation is generated by mixing defined volumes of aqueous solutions comprising suitable constituents at suitable concentrations. For example, a formulation typically contains the Fc-EPO protein at a concentration from 0.1 to 200 mg/ml, preferably from 0.2 to 10 mg/ml, more preferably from 0.5 to 6 mg/ml.

Buffer components include any physiologically compatible substances that are capable of regulating pH, such as, for example, citrate salts, acetate salts, histidine salts,

succinate salts, maleate salts, phosphate salts, lactate salts, their respective acids or bases or mixtures thereof. Commonly used buffer components are citrate salts and/or their free acid. A formulation typically contains a buffer component at a concentration from 10 to 100 mmol/l, preferably from 2 to 20 mmol/l, more preferably 10 mmol/l.

Surfactants for Fc-EPO formulations can be any excipient used as surfactants in pharmaceutical compositions, preferably polyethylene-sorbitane-esters (Tweens®), such as, Polyoxyethylene(20)-sorbitanmonolaurate, Polyoxyethylene(20)-sorbitanemonopalmitate and Polyoxyethylene(20)-sorbitanemonostearate, and polyoxytheylene-polyoxypropylene-copolymers. A formulation typically contains a surfactant at a concentration from 0.001 to 1.0 % w/v, preferably from 0.005 to 0.1 % w/v, more preferably from 0.01 to 0.5 % w/v.

A formulation can also contain one or more amino acids. Suitable amino acids include, but are not limited to, arginine, histidine, ornithine, lysine, glycine, methionine, isoleucine, leucine, alanine, phenylalanine, tyrosine, and tryptophan. In one embodiment, a formulation of Fc-EPO contains glycine. Preferably, amino acids are used in salt forms, for example, a hydrochloride salt. Applicable amino acid concentrations range from 2 to 200 mmol/L, or from 50 to 150 mmol/L.

Additionally, a formulation can contain sugars such as sucrose, trehalose, sorbitol; antioxidants such as ascorbic acid or glutathion; preservatives such as phenol, m-cresol, methyl- or propylparabene; chlorbutanol; thiomersal; benzalkoniumchloride; polyethyleneglycols; cyclodextrins and other suitable components.

It is desirable that an Fc-EPO formulation is isotonic. For example, osmolality of a formulation can range from 150 to 450 mOsmol/kg. Pharmaceutical formulations have to be stable for the desired shelf-life at the desired storage temperature, such as at 2-8°C, or at room temperature. A useful formulation containing an Fc-EPO protein is well tolerated physiologically, easy to produce, can be dosed accurately, and is stable during storage at 2°C - 8°C or 25°C, during multiple freeze-thaw cycles and mechanical stress, as well as other stresses such as storage for at least 3 months at 40°C. The stability of Fc-EPO formulations can be tested in a stress test. An exemplary stress test is described in Example 13.

Administration

The therapeutic compositions containing Fc-EPO fusion proteins produced according to the present invention can be administered to a mammalian host by any route.

Thus, as appropriate, administration can be oral or parenteral (*e.g.*, *i.v.*, *i.a.*, *s.c.*, *i.m.*), including intravenous and intraperitoneal routes of administration. In addition, administration can be by periodic injections of a bolus of the therapeutics or can be made more continuous by intravenous or intraperitoneal administration from a reservoir which is external (*e.g.*, an i.v. bag). In certain embodiments, the therapeutics of the instant invention can be pharmaceutical-grade. That is, certain embodiments comply with standards of purity and quality control required for administration to humans. Veterinary applications are also within the intended meaning as used herein.

The formulations, both for veterinary and for human medical use, of the therapeutics according to the present invention typically include such therapeutics in association with a pharmaceutically-acceptable carrier and optionally other ingredient(s). The carrier(s) can be "acceptable" in the sense of being compatible with the other ingredients of the formulations and not deleterious to the recipient thereof. Pharmaceutically acceptable carriers, in this regard, are intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds also can be incorporated into the compositions. The formulations can conveniently be presented in dosage unit form and can be prepared by any of the methods well known in the art of pharmacy/microbiology. In general, some formulations are prepared by bringing the therapeutics into association with a liquid carrier or a finely divided solid carrier or both, and then, if necessary, shaping the product into the desired formulation.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include oral or parenteral, *e.g.*, intravenous, intradermal, inhalation (*e.g.*, after nebulization), transdermal (topical), transmucosal, nasal, buccal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as

ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide.

A preferred method for administration of Fc-EPO protein products of the invention is by parenteral (*e.g.*, IV, IM, SC, or IP) routes and the compositions administered would ordinarily include therapeutically effective amounts of product in combination with acceptable diluents, carriers and/or adjuvants. Effective dosages are expected to vary substantially depending upon the condition treated but therapeutic doses are presently expected to be in the range of 0.2 to 2 mcg/kg body weight of the active material. Standard diluents such as human serum albumin are contemplated for pharmaceutical compositions of the invention, as are standard carriers such as saline. Adjuvant materials suitable for use in compositions of the invention include compounds independently noted for erythropoietic stimulatory effects, such as testosterones, progenitor cell stimulators, insulin-like growth factor, prostaglandins, serotonin, cyclic AMP, prolactin and triiodothyronine, as well as agents generally employed in treatment of aplastic anemia, such as methenolene, stanozolol and nandrolone. *See, e.g.*, Resegotti, *et al.* (1981), Panminerva Medics, 23, 243-248; McGonigle, *et al.*, (1984) Kidney Int., 25(2), 437-444; Pavlovic-Kantera, *et al.*, (1980) Expt. Hematol., 8(Supp. 8), 283-291; and Kurtz, (1982) FEBS Letters, 14a(1), 105-108.

Also contemplated as adjuvants are substances reported to enhance the effects of, or synergize with, Fc-EPO, such as the adrenergic agonists, thyroid hormones, androgens and BPA as well as the classes of compounds designated "hepatic erythropoietic factors" (see, Naughton *et al.*, (1983) Acta. Haemat., 69, 171-179) and "erythrotropins" as described by Congote *et al.* in Abstract 364, Proceedings 7th International Congress of Endocrinology, Quebec City, Quebec, Jul. 1-7, 1984; Congote (1983), Biochem. Biophys. Res. Comm., 115(2), 447-483; and Congote, (1984), Anal. Biochem., 140, 428-433, and "erythrogenins" as described in Rothman, *et al.*, (1982), J. Surg. Oncol., 20, 105-108.

Useful solutions for oral or parenteral administration can be prepared by any of the methods well known in the pharmaceutical art, described, for example, in Remington's Pharmaceutical Sciences, (Gennaro, A., ed.), Mack Pub., 1990. Formulations for parenteral administration also can include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal

administration. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Suppositories for rectal administration also can be prepared by mixing the drug with a non-irritating excipient such as cocoa butter, other glycerides, or other compositions that are solid at room temperature and liquid at body temperatures. Formulations also can include, for example, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. Formulations for direct administration can include glycerol and other compositions of high viscosity. Other potentially useful parenteral carriers for these therapeutics include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration can contain as excipients, for example, lactose, or can be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Retention enemas also can be used for rectal delivery.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition can be sterile and can be fluid to the extent that easy syringability exists. It can be stable under the conditions of manufacture and storage and can be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, and sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filter sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation include vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

In one embodiment, the therapeutics are prepared with carriers that will protect against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials also can be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811. Microsomes and microparticles also can be used.

Oral or parenteral compositions can be formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Determining therapeutically-effective amount of Fc-EPO and dosing frequency

Generally, the therapeutics containing Fc-EPO fusion proteins produced according to the present invention can be formulated for parenteral or oral administration to humans or other mammals, for example, in therapeutically effective amounts, *i.e.*, amounts which provide appropriate concentrations of the drug to a target tissue for a time sufficient to

induce the desired effect. More specifically, as used herein, the term "therapeutically effective amount" refers to an amount of Fc-EPO fusion proteins giving an increase in hematocrit to a target hematocrit, or to a target hematocrit range that provides benefit to a patient or, alternatively, maintains a patient at a target hematocrit, or within a target hematocrit range. The amount will vary from one individual to another and will depend upon a number of factors, including the overall physical condition of the patient, severity and the underlying cause of anemia and ultimate target hematocrit for the individual patient. A target hematocrit is typically at least about 30%, or in a range of 30%-38%, preferably above 38% and more preferably 40%-45%. General guidelines relating to target hematocrit ranges for rHuEpo are also found in the EPOGEN® package insert dated 12/23/96 and are 30%-36%, or alternatively 32%-38% as stated therein. It is understood that such targets will vary from one individual to another such that physician discretion may be appropriate in determining an actual target hematocrit for any given patient. Nonetheless, determining a target hematocrit is well within the level of skill in the art.

A therapeutically effective amount of an Fc-EPO protein may be readily ascertained by one skilled in the art. Example 15 sets forth a clinical protocol which has as one objective to determine a therapeutically effective amount of an Fc-EPO in once per week, once per two weeks, and once per month dosing. For example, a dose range for once per week or once per two weeks administration is from about 0.075 to about 4.5 mcg Fc-EPO per kg per dose. A dose range for once per month administration is 0.45 to 4.5 mcg Fc-EPO per kg per dose.

The effective concentration of the Fc-EPO fusion protein of the invention that is to be delivered in a therapeutic composition will vary depending upon a number of factors, including the final desired dosage of the drug to be administered and the route of administration. The preferred dosage to be administered also is likely to depend on such variables as the type and extent of disease or indication to be treated, the overall health status of the particular patient, the relative biological efficacy (*e.g.*, level of sialylation) of the therapeutics delivered, the formulation of the therapeutics, the presence and types of excipients in the formulation, and the route of administration. In some embodiments, the therapeutics of this invention can be provided to an individual using typical dose units deduced from the mammalian studies using non-human primates and rodents. As described above, a dosage unit refers to a unitary dose which is capable of being administered to a patient, and which can be readily handled and packed, remaining as a

physically and biologically stable unit dose comprising either the therapeutics as such or a mixture of it with solid or liquid pharmaceutical diluents or carriers.

The dosing frequency for a therapeutic containing the Fc-EPO fusion protein will vary depending upon the condition being treated and the target hematocrit, but in general will be less than three times per week. The dosing frequency may be about once or twice per week. The dosing frequency may also be less than about one time per week, for example about once every two weeks (about one time per 14 days), once per month or once every two months. It is understood that the dosing frequencies actually used may vary somewhat from the frequencies disclosed herein due to variations in responses by different individuals to the erythropoietin and its analogs; the term "about" is intended to reflect such variations.

The invention also provides for administration of a therapeutically effective amount of iron in order to maintain increased erythropoiesis during therapy. The amount to be given may be readily determined by one skilled in the art based upon therapy with rHuEpo. Additionally, the therapeutics of the present invention can be administered alone or in combination with other molecules known to have a beneficial effect on the particular disease or indication of interest. By way of example only, useful cofactors include symptom-alleviating cofactors, including antiseptics, antibiotics, antiviral and antifungal agents and analgesics and anesthetics.

Prodrug

Therapeutics of the invention also include the "prodrug" derivatives. The term prodrug refers to a pharmacologically inactive (or partially inactive) derivative of a parent molecule that requires biotransformation, either spontaneous or enzymatic, within the organism to release or activate the active component. Prodrugs are variations or derivatives of the therapeutics of the invention which have groups cleavable under metabolic conditions. Prodrugs become the therapeutics of the invention which are pharmaceutically active *in vivo*, when they undergo solvolysis under physiological conditions or undergo enzymatic degradation. A prodrug of this invention can be called single, double, triple, and so on, depending on the number of biotransformation steps required to release or activate the active drug component within the organism, and indicating the number of functionalities present in a precursor-type form. Prodrug forms often offer advantages of solubility, tissue compatibility, or delayed release in the mammalian organism (see, Bundgard, (1985) Design of Prodrugs, pp. 7-9, 21-24,

Elsevier, Amsterdam; Silverman, (1992) The Organic Chemistry of Drug Design and Drug Action, pp. 352-401, Academic Press, San Diego, Calif.). Moreover, the prodrug derivatives according to this invention can be combined with other features to enhance bioavailability.

In vivo expression

The Fc-EPO fusion protein of the present invention can be provided by *in vivo* expression methods. For example, a nucleic acid encoding an Fc-EPO fusion protein can be advantageously provided directly to a patient suffering from a hematopoietic disorders or deficiency, or may be provided to a cell *ex vivo*, followed by administration of the living cell to the patient. *In vivo* gene therapy methods known in the art include providing purified DNA (*e.g.* as in a plasmid) , providing the DNA in a viral vector, or providing the DNA in a liposome or other vesicle (see, for example, U.S. Patent No. 5,827,703, disclosing lipid carriers for use in gene therapy, and U.S. Patent No. 6,281,010, providing adenoviral vectors useful in gene therapy).

Methods for treating disease by implanting a cell that has been modified to express a recombinant protein are also known. See, for example, U.S. Patent No. 5,399,346, disclosing methods for introducing a nucleic acid into a primary human cell for introduction into a human.

In vivo expression methods are particularly useful for delivering a protein directly to targeted tissues or cellular compartment without purification. In the present invention, gene therapy using the sequence encoding Fc-EPO can find use in a variety of disease states, disorders and states of hematologic irregularity including anemia, in particularly correction of anemia of a type associated with chronic renal failure and the like. A nucleic acid sequence coding for an Fc-EPO fusion protein can be inserted into an appropriate transcription or expression cassette and introduced into a host mammal as naked DNA or complexed with an appropriate carrier. Monitoring of the production of active Fc-EPO protein can be performed by nucleic acid hybridization, ELISA, western hybridization, and other suitable methods known to ordinary artisan in the art.

It has been found that a plurality of tissues can be transformed following systemic administration of transgenes. Expression of exogenous DNA following intravenous injection of a cationic lipid carrier/exogenous DNA complex into a mammalian host has been shown in multiple tissues, including T lymphocytes, reticuloendothelial system,

cardiac endothelial cells lung cells, and bone marrow cells, *e.g.*, bone marrow-derived hematopoietic cells.

The *in vivo* gene therapy delivery technology as described in U.S. Patent No. 6,627,615, is non-toxic in animals and transgene expression has been shown to last for at least 60 days after a single administration. The transgene does not appear to integrate into host cell DNA at detectable levels *in vivo* as measured by Southern analysis, suggesting that this technique for gene therapy will not cause problems for the host mammal by altering the expression of normal cellular genes activating cancer-causing oncogenes, or turning off cancer-preventing tumor suppressor genes.

Examples

Example 1. Constructs encoding Fc-EPO fusion proteins

Plasmid phC10-Fcg2h(FN→AQ)-M1-EPO encoding an Fc-EPO fusion protein containing a normal erythropoietin portion and plasmid phC10-Fcg2h(FN→AQ)-M1-EPO(NDS) encoding an Fc-EPO fusion protein with NDS mutations were constructed as follows.

The nucleic acid sequence encoding human erythropoietin was codon-optimized for high expression in mammalian cells. For example, SEQ ID NO:3 shows an example of coding sequences of mature human erythropoietin with modified codons to optimize translation. The sequence of the 5' end was also modified to include a Sma I site to facilitate subcloning.

SEQ ID NO:3

CCCGGGtGCCCCACCACGCCTCATCTGTGACAGCCGAGTgCTGGAGAGGTACCTCTTGGAGGCCA
AGGAGGCCGAGAATATCACGACcGGCTGTGCTGAACACTGCAGCTTGAATGAGAAcATCACcGTg
CCtGACACCAAAGTgAATTTCTATGCCTGGAAGAGGATGGAGGTtGGcCAGCAGGCCGTAGAAGT
gTGGCAGGGCCTGGCCCTGCTGTCGGAAGCTGTCCTGCGGGGCCAGGCCCTGTTGGTCAACTCTT
CCCAGCCGTGGGAGCCCCTGCAaCTGCATGTGGATAAAGCCGTgAGTGGCCTTCGCAGCCTCACC
ACTCTGCTTCGGGCTCTGgGAGCCCAGAAGGAAGCCATCTCCCCTCCAGATGCGGCCTCAGCTGC
TCCcCTCCGcACAATCACTGCTGACACTTTCCGCAAACTCTTCCGAGTCTACTCCAATTTCCTCC
GGGGAAAGCTGAAGCTGTACACAGGGGAGGCCTgcCGGACAGGGGACAGATGActcgag

(Small characters indicate base differences from the wild-type human erythropoietin coding sequence. The changes are predicted to increase the expression level in mammalian cells but not to change the expressed protein sequence.)

NDS mutations were introduced into the erythropoietin portion by site-directed mutagenesis as described in PCT publication WO 01/36489, the disclosures of which are hereby incorporated by reference. For example, an Xma I-Xho I DNA fragment containing a form of the human erythropoietin coding sequence with mutations resulting in the amino acid substitutions His32Gly, Cys33Pro, Trp88Cys, and Pro90Ala, as disclosed in WO01/36489, was used. The corresponding protein sequence is shown in SEQ ID NO:4.

APPRLICDSRVLERYLLEAKEAENITTGCAEGPSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQ
GLALLSEAVLRGQALLVNSSQPCEGLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPDAASAAPL
RTITADTFRKLFRVYSNFLRGKLKLYTGEACRTGDR (SEQ ID NO:4)

A hybrid Fc portion, including an IgG2-derived CH2 domain and an IgG1-derived hinge region, was constructed as described in U.S. Patent Publication No. 20020147311 and, for, example, in WO 01/058957.

The Xma I-Xho I DNA fragment encoding a form of erythropoietin was inserted into a plasmid vector, for example, pdCs-Fc-X, that encodes an altered hinge region from IgG1 and a CH2 and CH3 region from IgG2, except that there were two sets of mutations (referred to herein as M1 set mutations) that resulted in amino acid substitutions in the region of the CH3 C-terminus, such that the sequence at the junction of the CH3 C-terminus and the EPO N-terminus is as follows:

.... TQKSATATPGA-APPRLI ....(SEQ ID NO:5)

The first set of mutations, which change the sequence KSLSLSPG (SEQ ID NO:6) of the IgG2 CH3 region to KSATATPG (SEQ ID NO:7), is disclosed in WO 02/079232. The effect of the substitution of Leu-Ser-Leu-Ser (position 3 to position 6 of SEQ ID NO:6) with Ala-Thr-Ala-Thr (position 3 to position 6 of SEQ ID NO:7) is to remove potential human non-self T-cell epitopes that may arise because the junction between human Fc and human erythropoietin contains non-self peptide sequences. The second set consisting of the single amino acid substitution K to A at the C-terminal amino acid of the CH3 region, is disclosed in WO 01/58957.

Expression vector pdCs-Fc-X for the expression of Fc fusion proteins was described by Lo *et al.*, (1998) Protein Engineering 11:495. The plasmid phC10-Fc-X was constructed from pdCs-Fc-X by replacing the coding region of the dihydrofolate reductase (DHFR) gene conferring resistance to methotrexate with the gene conferring

resistance to Hygromycin B. A Nhe I/Nsi I Hygromycin B DNA fragment was obtained by PCR amplification of the Hygromycin B gene from the template plasmid pCEP4 (Invitrogen) using the primers 5'-GCTAGCTTGGTGCCCTCATGAAAAAGCCTGAACTC-3' (SEQ ID NO:8) and 5'-ATGCATTCAGTTAGCCTCCCCCATC-3' (SEQ ID NO:9). The PCR fragment was cloned into the TA cloning vector pCR2.1 (Invitrogen), and its sequence confirmed.

Plasmid phC10-Fcg2h-M1-EPO(NDS) was generated by a triple ligation of Nhe I/Afl I and Afl II/Nsi I DNA fragments from pdCs-Fcg2h-M1-EPO(NDS) and the Nhe I/Nse I Hygromycin B fragment.

Additionally, a mutation leading to a double amino acid substitution, "FN>AQ", within the Gln-Phe-Asn-Ser amino acid sequence within the CH2 domain of the IgG2 heavy chain that eliminates a potential T-cell epitope and N-linked glycosylation in the Fc portion was introduced by PCR mutagenesis. The mutagenic primers 5'-AGCAGGCCCAGAGCACGTTCCGTGTGGT-3' (SEQ ID NO:10) and 5'-GAACGTGCTCTGGGCCTGCTCCTCCCGT-3' (SEQ ID NO:11) were paired respectively with a downstream primer containing a Sac II site 5'-CCCCGCGGGTCCCACCTTTGG-3' (SEQ ID NO:12) and an upstream primer containing a Pvu II site 5'-CCCAGCTGGGTGCTGACACGT-3' (SEQ ID NO:13), and two overlapping DNA fragments were amplified from the template DNA pdC10-Fcg2h-M1-EPO(NDS). In a second amplification round, a Pvu II/Sac II fragment containing the mutation (FN→AQ) was amplified using the upstream primer (SEQ ID NO:13) and downstream primer (SEQ ID NO:12) from the PCR products from the first amplification round. The Pvu II/Sac II fragment was cloned into a TA vector pCR2.1 (Invitrogen), and its sequence verified. Construct pdC10-Fcg2h(FN>AQ)-M1-EPO(NDS) was generated from a triple ligation of the Pvu II/Sac II fragment, a Xho I/Sac II fragment from pdC10-Fcg2h-M1-EPO, and a Xho I/Pvu II fragment from pdC10-Fcg2h-M1-EPO(NDS).

To introduce the FN>AQ mutation into the plasmid phC10-Fcg2h-M1-EPO, the appropriate DNA fragments from phC10-Fcg2h-M1-EPO and from pdC10-Fcg2h(FN→AQ)-M1-EPO were combined. Both phC10-Fcg2h-M1-EPO and pdC10-Fcg2h(FN→AQ)-M1-EPO constructs were digested with Xho I and Xba I, and the 5.7 kb Xho I/Xba I phC10-Fcg2h-M1-EPO(NDS) fragment was ligated with the 1.9 kb pdC10-Fcg2h(FN→AQ)-M1-EPO fragment, generating phC10-Fcg2h(FN→AQ)-M1-EPO.

To introduce the FN→AQ mutation into the plasmid phC10-Fcg2h-M1-EPO(NDS), the two appropriate Xho I/Sma I digested fragments from phC10-Fcg2h-M1-EPO(NDS) and from phC10-Fcg2h(FN→AQ)-M1-EPO were ligated together, generating phC10-Fcg2h(FN→AQ)-M1-EPO(NDS).

The amino acid sequence of Fc-EPO encoded by pdC10-huFcg2h(FN→AQ)-M1-EPO is shown in SEQ ID NO:14.

EPKSSDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDG
VEVHNAKTKPREEQAQSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTK*GQPREP*
QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLT
*VDKSRWQQGNVFSCSVMHEALHNHYTQKSATATPG*AAPPRLICDSRVLERYLLEAKEAENITTGC
AEHCSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSEAVLRGQALLVNSSQPWEPLQLH
VDKAVSGLRSLTTLLRALGAQKEAISPPDAASAAPLRTITADTFRKLFRVYSNFLRGKLKLYTGE
ACRTGDR (SEQ ID NO:14)

The amino acid sequence of Fc-EPO(NDS) encoded by pdC10-huFcg2h(FN→AQ)-M1-EPO(NDS) is shown in SEQ ID NO:15.

EPKSSDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDG
VEVHNAKTKPREEQAQSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTK*GQPREP*
QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLT
*VDKSRWQQGNVFSCSVMHEALHNHYTQKSATATPG*AAPPRLICDSRVLERYLLEAKEAENITTGC
AEGPSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSEAVLRGQALLVNSSQPCEALQLH
VDKAVSGLRSLTTLLRALGAQKEAISPPDAASAAPLRTITADTFRKLFRVYSNFLRGKLKLYTGE
ACRTGDR (SEQ ID NO:15)

The underlined sequence track represents the EPO portion, the double-underlined sequence represents the IgG1 hinge, and the non-underlined sequence represents the CH2 and CH3 domain of the modified IgG chain, wherein the italics written sequence represents the CH3 domain.

Example 2. Expression of Fc-EPO in various cell lines

For rapid analysis of the fusion protein, a plasmid, phC10-Fcg2h(FN→AQ)-M1-EPO(NDS) or phC10-Fcg2h(FN→AQ)-M1-EPO, was introduced into suitable tissue culture cells by standard transient transfection methods, such as, for example, by calcium phosphate-mediated DNA co-precipitation (Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press), or by lipofection using Lipofectamine Plus (Life Technologies) according to the manufacturer's protocol.

In order to obtain stably transfected BHK-21 cells, a plasmid, phC10-Fcg2h(FN→AQ)-M1-EPO(NDS) or phC10-Fcg2h(FN→AQ)-M1-EPO, was introduced into BHK-21 cells by electroporation. For high-efficiency electroporation, BHK-21 cells, grown in MEM medium (supplemented with non-essential amino acids and sodium pyruvate as recommended by the American Type Culture Collection (ATCC)), were washed once with PBS; and approximately $5x10^6$ cells were resuspended in 0.5 ml PBS and incubated with 10 μg of linearized plasmid DNA in a Gene Pulser™ Cuvette with a 0.4 cm electrode gap (BioRad, Hercules, CA) on ice for 10 min. Electroporation was performed using a Gene Pulser™ (BioRad, Hercules, CA) with settings at 0.25 V and 500 μF. Cells were allowed to recover for 10 min on ice, resuspended in growth medium, and plated onto two 96 well plates. Hygromycin B (Hyg B) was added to the growth medium two days post-transfection at a concentration of 300 micrograms/ml. The cells were fed every 3 days for two to three more times, and Hyg B resistant stable clones appeared in 2 to 3 weeks.

To identify stable clones producing high levels of the Fc-EPO fusion protein, supernatants from clones were assayed by ELISA with anti-Fc antibodies. High-producing clones were isolated and propagated in growth medium containing 300 micrograms/ml Hyg B. For protein production purposes, BHK-21 cells were routinely grown in a supplemented DMEM/F-12 medium, or in another suitable medium such as VP-SFM (Life Technologies). The Fc-EPO fusion protein was harvested from the conditioned medium by standard normal-flow filtration, and the clarified material was stored at 4 degrees Celsius until further purification. Typically, in a roller bottle production mode, yields of 6 – 12 mcg/ml of Fc-EPO proteins were obtained from BHK-21 cells.

Fc-EPO fusion proteins were also expressed in and recovered from NS/0 cells. NS/0 clones stably maintaining the plasmid pdC10-Fcg2h(FN→AQ)-M1-EPO or pdC10-Fcg2h(FN→AQ)-M1-EPO(NDS) were established by methods previously described in PCT publication WO 01/36489, the entire disclosures of which are hereby incorporated by reference. Typically, yields of 50 – 100 mcg/ml of Fc-EPO protein were obtained from NS/0 cells.

Example 3. Adaptation of BHK cells for growth in suspension and/or in protein-free media

BHK is an adherent cell line commonly grown in serum-containing media, such as, for example, MEM + 10 % heat-inactivated fetal bovine serum (FBS). To maintain and expand BHK cells, they are periodically (*e.g.*, in 4 day intervals) detached from their substrate, typically by the action of a trypsin-EDTA solution, diluted in fresh media and re-seeded in appropriate vessels. However, BHK cells can be adapted for growth in suspension and in serum-free and/or protein-free media by the following procedures.

In a typical adaptation process, BHK cells were first cultured in 75:25 (v/v) mixture of MEM+FBS : target medium until exponential stage, and subsequently subcultured at an appropriate cell density in 50:50 (v/v), 25:75 (v/v), and finally 0:100 (v/v) original medium : target medium. During the adaptation process, the growth of the BHK cells was monitored by visual inspection. The following serum-free media were tested for adaptation: 293 SFM II (Invitrogen Corp., cat # 11686-929), CHO-S-SFM II (Invitrogen Corp., cat # 12052-098), VP-SFM (Invitrogen Corp., cat # 11681-020), Opti-Pro SFM (Invitrogen Corp., cat # 12309), CD Hybridoma (Invitrogen Corp., cat # 11279-023), and H-SFM (Invitrogen Corp., cat # 12045-076).

To switch BHK cells from an adherent cell line to a suspension cell line during the adaptation process, the culture mix was allowed to sit before each passage, and the top 25% of the cell suspension was removed and diluted into a fresh medium. Because cells that aggregate settled to the bottom of the culture vessels more rapidly than single and doublet cells, the top 25% cell suspension generally contains those cells that exhibit the least amount of aggregation. Thus, each passage expands and enriches the BHK cells less prone to aggregation, and suspension cell lines of BHK clones expressing Fc-EPO proteins were established by this method.

It was found that BHK cells expressing Fc-EPO proteins could be adapted for growth in VP-SFM or Opti-PRO SFM serum-free media and suspension cultures were obtained. The BHK cells expressing Fc-EPO fusion proteins were not able to grow in the following serum-free media: 293 SFM II, CHO-S-SFM II, CD Hybridoma, and H-SFM.

BHK cells adapted to the serum-free medium, VP-SFM, were further adapted to grow in a protein-free medium, *e.g.*, DMEM/F-12 (Invitrogen Corp., cat # 11039-021) by sequentially culturing the BHK cells, at an appropriate cell density, in 75:25 (v/v), 50:50 (v/v), 25:75 (v/v), and finally 0:100 (v/v) VP-SFM : DMEM/F-12 mixture. The protein-

free medium DMEM/F-12 was supplemented with Glutamine (6 mM final), 2 g/l HyPep 4601 (Quest International, Chicago, IL, cat # 5Z10419,), 2 g/l HyPep 1510 (Quest International, Chicago, IL, cat # 5X59053,), 10 µl/l (v/v) Ethanolamine (Sigma, cat# E0135), and 5 µM Tropolone (Sigma, cat # T7387). A BHK cell line stably expressing Fc-EPO fusion protein competent to grow in supplemented DMEM/F-12 was obtained by this method and maintained at high cell viability.

Example 4. Purification and characterization of protein aggregation state

For analysis, Fc-EPO fusion proteins were purified from cell-culture supernatants via Protein A chromatography based on the affinity of the Fc portion for Protein A. The conditioned supernatant from cells expressing Fc-EPO proteins was loaded onto a pre-equilibrated Fast-Flow Protein A Sepharose column. The column was washed extensively with sodium phosphate buffer (150 mM Sodium Phosphate, 100 mM NaCl at neutral pH). Bound protein was eluted by a low pH (pH 2.5 - 3) sodium phosphate buffer (composition as above) and the eluted fractions were immediately neutralized.

To assess the aggregation state of the Fc-EPO fusion proteins produced by different cell lines, Protein A purified samples were analyzed by analytical size exclusion chromatography (SEC). The samples were fractionated by HPLC-SEC (*e.g.*, Super 3000 SW, TosoHaas, Montgomeryville, PA), in a fifteen-minute run at a flow rate of 0.35 ml/min. A substantial portion of the Fc-EPO proteins (*e.g.*, up to 90% to 100% of total yield) produced from BHK cells was non-aggregated. Furthermore, samples of the Fc-EPO fusion proteins analyzed by reducing SDS-PAGE (precast NuPAGE 4% - 12% gel, NuPAGE, Novex) revealed substantially a single band, indicating that the products were resistant to degradation under standard operating procedures.

Fc-EPO fusion proteins purified from BHK cells grown in suspension, in serum-free media, and/or in protein-free media were also characterized by SDS-PAGE and analytical SEC as described above. The proteins were found to be substantially non-aggregated and not degraded, like proteins synthesized in BHK cells grown in serum-containing media.

Example 5A. Characterization of glycosylation patterns

Serine126 in human erythropoietin is in a sequence compatible with O-glycosylation, and is conserved in all mammalian erythropoietin proteins. However, serine126 is in a "floppy loop" that does not pack tightly against the rest of the protein.

In the absence of O-glycosylation, this region of erythropoietin might be particularly sensitive to proteolysis.

The status of O-glycosylation at Ser126 in Fc-EPO proteins produced in different cell lines was examined by reversed phase HPLC. Samples were denatured and reduced, diluted into 0.1% triflouroacetic acid (TFA), and injected into a reversed phase HPLC column (*e.g.*, a Vydac C4 column, Grace Vydac). A gradient into 0.085% TFA in acetonitrile was applied and the retention times of the protein samples were recorded. It was found that Fc-EPO and Fc-g2h(FN→AQ)-EPO synthesized in BHK-21 cells produced two partially overlapping major peaks (Peak #1 and Peak #2). The peak fractions were further analyzed by peptide mapping. It was found that Peak #1 corresponded to a form of Fc-EPO that was glycosylated at Ser126, as indicated by the absence of a signature peptide (Peptide #36), whereas Peak #2 corresponded to a form of Fc-EPO that was not glycosylated at Ser126, as indicated by the presence of the signature peptide (Peptide #36). It was found that Ser126 is modified by O-glycosylation in about 60% of the Fc-EPO molecules produced from BHK cells, which is consistent with what has been reported for naturally occuring EPO. Furthermore, growth of BHK cells in supplemented protein-free DMEM/F-12 medium had a positive effect on frequency of O-glycosylation.

Example 5B. Characterization of sialylation patterns

The extent of sialylation of Fc-EPO fusion proteins synthesized in NS/0, BHK, 293, and PerC6 cells was compared by isoelectric focusing (IEF) gel electrophoresis. Briefly, samples, concentrated to 2 mg/ml and desalted if necessary, were added to an equal volume of IEF Sample Buffer pH 3-7, and run on a vertical precast Novex pH 3-7 IEF Gel (Novex, cat# EC6655B/B2) for 2.5 hours, first hour at 100V, second hour at 200V and last 30 minutes at 500V. The gel was then fixed, stained and destained.

In one particular experiment, the following samples were compared (samples were derived from cells grown in serum-containing media):

1. Fcg2h-EPO(NDS) from NS/0
2. Fcg2h-EPO(NDS) from BHK-21
3. Fcg2h-EPO from BHK-21
4. Fcg2h("Delta Lys")-EPO from BHK-21
5. Fcg4h(FN→AQ "Delta Lys")-EPO from BHK-21
6. Fcg4h("Delta Lys")-EPO from BHK-21

In this group, "Delta Lys" refers to a deletion of the lysine at the C-terminus of the Fc domain (samples 4-6). Samples 1-3 have a mutation of this C-terminal lysine to an alanine. Therefore this C-terminal lysine is absent in all of the samples and there is no resulting charge difference between the samples. All cells were grown as adherent cells in serum-containing media.

Samples were loaded onto a pH 3-7 IEF gel and compared with standards that focused at pH 3.5, 4.2, 4.5, 5.2, 5.3, 6.0, and 6.9 (Serva Electrophoresis, Germany). The first sample, Fcg2h-EPO(NDS) from NS/0, migrated as a distribution of bands with isoelectric points between about pH 5.3 and 6.5; the most intense bands were present at pH 6.0-6.1. The second sample, Fcg2h-EPO(NDS) from BHK-21, ran as a distribution of intense bands with isoelectric points at about pH 4.6 to pH 5.0, with fainter bands from pH 5.0 to about pH 6.0; the most intense bands were present at pH 4.8-4.9. The third and fourth samples, Fcg2h-EPO from BHK-21 and Fcg2h("Delta Lys")-EPO from BHK-21, respectively, both had a distribution of bands from about pH 4.7 to 6.0 with the most intense bands focused at about pH 5.3. The fifth and sixth samples, Fcg4h(FN→AQ "Delta Lys")-EPO from BHK-21 and Fcg4h("Delta Lys")-EPO from BHK-21, respectively, had a focusing pattern similar to that of the second sample, *i.e.*, ran as a distribution of intense bands with isoelectric points at about pH 4.6 to pH 5.0, with fainter bands from pH 5.0 to about pH 6.0. These results indicate that synthesis of Fc-EPO fusion proteins in BHK cells generally resulted in a significantly more acidic product than identical or similar products synthesized in NS/0 cells.

In other experiments, samples of Fcg2h-M1-EPO(NDS) from BHK cells were treated with neuraminidase, which removes sialic acid from oligosaccharides. The resulting neuraminidase-treated samples were run on an IEF gel and found to focus as a few bands at pH 6.9 and greater. When the banding patterns of samples from BHK cells with or without neuraminidase treatment and of samples from NS/0 cells were compared, about 27 distinct sialylated species were identified. The 27 species correspond well with the predicted 28 different species that could result from varying extents of sialylation of an Fc-EPO fusion protein in homodimeric configuration. According to this analysis, Fcg2h-EPO with 4-5 sialic acid residues focused with the pH 6.9 marker, and Fcg2h-EPO with 11-12 sialic acid residues focused with the pH 6.0 marker. It was found that a population of Fcg2h-EPO proteins synthesized in BHK cells appeared to have an average of 21 sialic acid residues per protein molecule. In contrast, a population of Fc(g2h)-EPO

proteins synthesized in NS/0 cells appeared to have an average about 10 sialic acid residues per protein molecule.

In subsequent experiments, BHK cells expressing Fc-EPO proteins were adapted to serum-free growth conditions and conditions appropriate for large-scale production, *e.g.*, suspension conditions. Fc-EPO proteins produced from BHK cells grown in serum-free and in suspension were analyzed by IEF gel electrophoresis as described above. These alterations in growth conditions resulted in shifts of, at most, only 0.1 to 0.3 pH units in the isoelectric point of the most intense band.

Samples of the Fc-EPO fusion proteins synthesized in supplemented DMEM/F-12 protein-free media were similarly characterized by IEF gel electrophoresis. It was found that the protein product was sialylated to a greater extent and exhibited more homogeneous sialylation than the corresponding product obtained from cells grown in serum-free media such as VP-SFM.

The extent of sialylation of Fc-EPO proteins produced in different cell lines was also qualitatively confirmed by lectin-binding studies. For example, Fc-EPO fusion proteins were first separated by standard SDS gel electrophoresis and blotted, then probed with modified lectins that recognize distinct carbohydrate moieties (*e.g.*, commercially available from Roche Applied Science, Indianapolis, IN), and bound lectins can be visualized. Suitable lectins include, but are not limited to, Sambucus nigra agglutinin (SNA) or Maackia amurensis agglutinin (MAA), which recognize sialic acids with specific linkages, and Datura stramonium agglutinin (DAA), Peanut agglutinin (PNA) and jacalin, which recognize other regions of the carbohydrate moiety such as the O-glycan core. Based on lectin binding assays, sialylation levels of Fc-EPO fusion proteins produced in different cell lines could be determined.

Example 6. *In vitro* biological activity of Fc-EPO variants

The *in vitro* activities of different Fc-EPO proteins were tested in a cell-based assay. The TF-1 cell line expresses EPO receptors, and accordingly, under appropriate culture conditions, its incorporation of tritiated thymidine is a function of EPO or EPO-like protein activity (Hammerlling *et al.*, (1996) J. Pharmaceutical and Biomedical Analysis, 14:1455; Kitamura *et al.*, (1989) J. Cellular Physiol., 140:323). Specifically, TF-1 cells in active log-phase were washed twice in a medium without EPO, and plated at about 10^4 cells/well in microtiter plates. The cells were then incubated in a medium with

a titrated dilution series of the Fc-EPO variants for 48 hours. 0.3 μCi of ^{3}H-thymidine were added to the wells ten hours before assaying cell proliferation. As controls, TF-1 cells were also incubated in the presence of recombinant human EPO, and hyperglycosylated EPO analogue Aranesp®. Incorporation of radioactive thymidine was measured as total TCA-precipitable counts. As shown in Table 2, the activities of Fcg2h-M1-EPO molecules are comparable to that of recombinant human EPO.

Some general conclusions can be drawn from this data. Consistent with previously reported results, EPO produced from CHO cells has an ED50 of about 0.7 ng/ml; this includes the NIBSC EPO standard, EPO from R&D Systems, and commercial Procrit®. Aranesp® is significantly less active *in vitro*, presumably reflecting its reduced on-rate due to its increased negative charges. Similarly, Fc-EPO produced from BHK cells is less active than Fc-EPO produced from NS/0 cells, which is consistent with the observation that Fc-EPO proteins produced from BHK cells are highly sialylated resulting in increased negative charges on the proteins.

Table 2

Protein	**ED50 (ng/ml)**	**S.D.**	**N**
EPO (NIBSC)	0.77	0.35	22
EPO (R&D Systems)	0.6	0.26	26
EPO (Procrit®)	0.68	0.15	6
EPO (Aranesp®)	2.4	0.96	10
Fcg2h-M1-EPO (NS/0)	0.35	0.15	14
Fcg2h-M1-EPO (BHK)	0.94	0.34	5

Example 7. Pharmacokinetic analysis of Fc-EPO variants

The pharmacokinetic profiles of different Fc-EPO proteins synthesized in various cell lines were characterized based on the following *in vivo* experiments. In one experiment, as shown in Figure 8, about 14 mcg of Fcg2h(N>Q)-EPO protein synthesized in NS/0 cells and in BHK cells were administered intravenously into Swiss-Webster mice. At various time points after administration (*e.g.*, T=0, ½, 1, 2, 4, 8, and 24 hours after administration), blood samples were collected and serum was prepared by centrifugation. The serum concentrations of Fc-EPO were determined by ELISA using anti-Fc antibodies. As shown in Figure 8, at 24 hours after administration, greater than 10% of the initial serum concentration of BHK-derived Fc-EPO remained in the serum, while less

than 0.1% of the initial serum concentration of the NS/0-derived Fc-EPO remained in the serum.

A similar experiment was done with Fcg2h-EPO(NDS) proteins synthesized in NS/0 cells and in BHK cells. About 14 mcg of Fcg2h-EPO(NDS) protein synthesized in NS/0 cells and in BHK cells were administered intravenously into Swiss-Webster mice. Blood samples were collected at T=0, ½, 1, 2, 4, 8, 24, and 36 hours after administration and the concentrations of Fcg2h-EPO(NDS) in serum were measured by anti-Fc ELISA. As shown in Figure 9, at 24 hours after administration, greater than 10% of the initial serum concentration of BHK-derived Fcg2h-EPO(NDS) remained in the serum, while less than 0.1% of the initial serum concentration of the NS/0-derived Fcg2h-EPO(NDS) remained in the serum.

Pharmacokinetic profiles of Fcg2h-EPO(NDS) produced in BHK-21 cells, PERC6 cells, and 293 cells were also compared. Specifically, a plasmid expressing Fcg2h-Epo(NDS) was transiently transfected into BHK, 293, and PERC6 cells. The expressed Fcg2h-Epo(NDS) fusion proteins were purified from different cell lines and were injected intravenously into Swiss-Webster mice at a concentration of 1.7 micrograms per mouse. Blood samples were taken at T=0, ½, 1, 2, 4, 8, 24, 48, and 72 hours, and the concentration of Fcg2h-Epo(NDS) in serum was measured by anti-Fc ELISA. As shown in Figure 10, at 24 hours after administration, greater than 10 % of the initial serum concentration of BHK-derived Fcg2h-EPO(NDS) remained in the serum, while less than 1% of the initial serum concentration of the 293 cell-derived Fcg2h-EPO(NDS) remained in the serum, and the PerC6 cell-derived Fcg2h-EPO(NDS) was almost undetectable in the serum. Similar results were obtained with Fcg2h(N→Q)-EPO proteins produced in BHK, PerC6, and 293 cells.

Similar experiments were conducted in mice to compare pharmacokinetic profiles of Fcg2h(N→Q)-EPO, Fcg2h-EPO(NDS), Fcg2h-EPO, and Aranesp® (*i.e.*, NESP). The Fc-EPO variants used herein were synthesized from BHK cells. It was observed that, at 48 hours after administration, less than 10% of the initial serum concentration of Aranesp® remained in serum, while greater than 10% of the initial serum concentrations of both Fcg2h(N→Q)-EPO and Fcg2h-EPO(NDS) remained in serum. These results indicate that Fcg2h(N→Q)-EPO and Fcg2h-EPO(NDS) proteins produced from BHK-21 cells have much longer serum half-lives than that of Aranesp®.

Example 8. *In vivo* potency of Fc-EPO variants

The *in vivo* biological activities of different Fc-EPO variants were measured by hematocrit (HCT) assays and reticulocyte assays in mice and rats.

In one HCT experiment, CD1 mice were injected intraperitoneally with Fcg2h(FN→AQ)-EPO proteins synthesized in BHK cells at dose 20 mcg/kg and 10 mcg/kg. Blood samples were taken from the mice at days 4, 7, 11, and 14, and centrifuged in capillary tubes. The amounts of sedimented RBCs were measured as fractions of the total volume. As illustrated in Figure 4, in response to the injection of Fcg2h(FN>AQ)-EPO proteins, the hematocrits increased dramatically first, then remained steady, finally decreasing.

In another experiment, Sprague-Dawley rats were injected intraperitoneally with the following proteins synthesized in BHK cells. All animals were dosed at 42.5 mcg/kg.

1. Fcg2h-EPO
2. Fcg2h-EPO(NDS)
3. Fcg4h-EPO
4. Fcg4h(N>Q)-EPO

HCT assays were performed with the blood samples taken from the injected mice as described above. As shown in Figure 5, in response to Fcg2h-EPO(NDS) and Fcg2h-EPO, the amount of hematocrits in the injected rats remained steady for an extended period of time, indicating that both Fcg2h-EPO(NDS) and Fcg2h-EPO proteins have prolonged serum half-lives and potent *in vivo* biological activity. It was also found that, as shown in Figure 5, Fcg4h-EPO and Fcg4h(N→Q)-EPO exhibited a shorter steady period and a faster decreasing of the serum concentration compared to Fcg2h-EPO(NDS) and Fcg2h-EPO proteins.

In another experiment, CD1 mice were administered intraperitoneally with the following samples.

1. Fcg2h-EPO(NDS) from BHK cells at doses of 85 mcg/kg, 42.5 mcg/kg, and 21.25 mcg/kg
2. Fcg2h-EPO(NDS) from NS/0 cells at doses of 85 mcg/kg, 42.5 mcg/kg, and 21.25 mcg/kg
3. Aranesp® (*i.e.*, NESP) at doses of 50 mcg/kg, 25 mcg/kg, and 12.5 mcg/kg

The protein amounts were calculated on the basis of protein molecular weight without carbohydrates. In this experiment, the molecular weight of Fcg2h-EPO(NDS)

protein is based on a monomer polypeptide. Accordingly, the ratio of molecular weights of Fcg2h-EPO(NDS) to NESP is about 1.71 to 1. Therefore, the dose ranges with each protein in this experiment were approximately equal.

As shown in Figure 6, Fcg2h-EPO(NDS) proteins synthesized in BHK cells exhibited the best hematocrit profile in terms of potency and duration of effect, indicating that Fcg2h-EPO(NDS) proteins from BHK cells have longer serum half-lives and more potent *in vivo* activities compared to both Fcg2h-EPO(NDS) from NS/0 cells and NESP. The hematocrit profiles of Fcg2h-EPO(NDS) from NS/0 cells and NESP are comparable.

Example 9. Comparison of Fc-EPO proteins with CH2-CH3 domains derived from IgG2 and from IgG4

A comparison of the cell-based erythropoietin activities of various Fc-EPO proteins revealed that fusion proteins with CH2 and CH3 domains derived from IgG4 were generally less active than corresponding proteins with CH2 and CH3 domains derived from IgG2. This conclusion is true for at least three types of Fc-EPO proteins, namely, proteins with the NDS mutations in the erythropoietin portion and synthesized in NS/0 cells (Table 3), proteins with the NDS mutations synthesized in BHK cells (Table 4), and proteins with normal erythropoietin synthesized in BHK cells (Table 5).

All of the proteins compared in the tables 3 to 5 below have a modified hinge derived from IgG1 and the M1 set of mutations at the C-terminus of the Fc portion. Activities of the proteins were determined by measuring the incorporation of tritiated thymidine into TF-1 cells stimulated by the proteins according to standard procedures described in Example 6. Activity is expressed as an ED50 in nanograms/ml of erythropoietin moieties.

Table 3: Cell-based activities of Fc-EPO fusion proteins with the NDS mutations and synthesized in NS/0 cells

Fc-EPO Proteins	ED50 (ng of EPO/ml)	S.D	No. Experiments
Fcg2h-M1-EPO(NDS) NS0 preparation 1	0.60	0.17	5
Fcg2h-M1-EPO(NDS) NS0 preparation 2	0.57	0.33	13
Fcg2h-M1-EPO(NDS) NS0 preparation 3	0.54	0.34	8
Fcg2h-M1-EPO(NDS) NS0 preparation 4	0.36	0.11	5
Fcg4h-M1-EPO(NDS) NS0 preparation 1	0.96	0.21	4

Table 4: Cell-based activities of Fc-EPO fusion proteins with the NDS mutations and synthesized in BHK cells

Fc-EPO Proteins	ED50 (ng of EPO/ml)	S.D.	No. Experiments
Fcg2h-M1-EPO(NDS) BHK preparation 1	0.81	0.23	11
Fcg2h-M1-EPO(NDS) BHK preparation 2	2.17	1.23	6
Fcg2h-M1-EPO(NDS) BHK preparation 3	1.16	0.28	5
Fcg2h-M1-EPO(NDS) BHK preparation 4	0.89	0.44	4
Fcg2h-M1-EPO(NDS) BHK preparation 5	1.09	0.41	4
Fcg4h-M1-EPO(NDS) BHK preparation 1	6.24	2.34	6

Table 5: Cell-based activities of Fc-EPO fusion proteins with wild-type EPO and synthesized in BHK cells

Fc-EPO Proteins	ED50 (ng of EPO/ml)	S.D.	No. Experiments
Fcg2h-M1-EPO BHK preparation 1	0.84	0.28	4
Fcg2h-M1-EPO BHK preparation 2	0.95	0.32	7
Fcg2h-M1-EPO BHK preparation 3	0.72	0.27	3
Fcg2h-M1-EPO BHK preparation 4	0.95	0.17	3
Fcg2h-M1-EPO BHK preparation 5	0.43	0.18	2
Fcg4h-M1-EPO BHK preparation 1	1.09	0.31	7
Fcg4h-M1-EPO BHK preparation 2	1.53	0.35	6

Activity data from *in vitro* cell-based assays usually can suggest pharmacokinetic profiles and *in vivo* potencies of erythropoietin-containing proteins. Generally, a decreased *in vitro* activity in a cell-based assay indicates a reduced on-rate for the EPO receptor, which correlates with improved pharmacokinetic properties (*e.g.*, extended half-life) and enhanced *in vivo* activity. However, the decreased *in vitro* activities of Fc-EPO fusion proteins with IgG4-derived CH2 and CH3 domains do not correlate with improved pharmacokinetics and enhanced *in vivo* biological activities. It was found that the pharmacokinetic profiles of Fc-EPO fusion proteins with IgG4-derived CH2 and CH3 domains were generally indistinguishable from the corresponding proteins with IgG2-derived CH2 and CH3 domains. It was also found that Fc-EPO fusion proteins with IgG4-derived CH2 and CH3 domains generally had less activity *in vivo* compared to the corresponding proteins with IgG2-derived CH2 and CH3 domains (see Figure 5).

Example 10. The effects of elimination of the glycosylation site in the Fc portion

Experiments were conducted to test the effects of elimination of the glycosylation site in the Fc portion on *in vitro* activity, pharmacokinetics, and *in vivo* potency. In particular, Fc-EPO fusion proteins containing either IgG2-derived CH2 and CH3 domains or IgG4-derived CH2 and CH3 domains were tested. The asparagine within the Gln-Phe-Asn-Ser amino acid sequence of IgG2 or IgG4, which corresponds to Asn297 of IgG1, was replaced with a glutamine. In most experiments, the phenylalanine with the Gln-Phe-Asn-Ser amino acid sequence was replaced with alanine to eliminate possible non-self T-cell epitopes that may result from the mutation of the asparagine. As shown in Table 6, in cell-based *in vitro* assays, the ED50 values of Fc-EPO proteins with the FN>AQ mutation eliminating the N-linked glycosylation site in the Fc portion are generally about 5-fold lower than that of Fc-EPO proteins without the mutation, indicating elimination of the N-linked glycosylation site resulted in a decreased *in vitro* activity in cell-based assays.

Experiments were also conducted to test the effects of elimination of the N-linked glycosylation on pharmacokinetics and *in vivo* potency. CD1 mice were treated with Fcg2h-M1-EPO, Fcg2h-M1-EPO(NDS), and Fcg2h(N>Q)-M1-EPO proteins synthesized in BHK cells at a dose of 42 mcg/kg each. It was observed that Fcg2h(N→Q)-M1-EPO protein showed better pharmacokinetic profile than the corresponding protein without N→Q mutation. Therefore, N>Q mutation, which eliminates the N-linked glycosylation in the IgG2-derived Fc portion, resulted in improved pharmacokinetics (*e.g.*, extended serum half-life). The extended serum half-life cannot be explained by an effect on binding to Fc receptors because IgG2-derived CH2 and CH3 domains already have essentially undetectable Fc-receptor binding.

Table 6: Elimination of the glycosylation site in the Fc portion reduces *in vitro* cell-based activity of the Fc-EPO fusion proteins.

Fc-EPO fusion proteins	ED50 (ng of EPO/ml)	S.D.	No. Experiments
Fcg2h-EPO BHK preparation 1	0.84	0.28	4
Fcg2h-EPO BHK preparation 2	0.95	0.32	7
Fcg2h-EPO BHK preparation 3	0.72	0.27	3
Fcg2h-EPO BHK preparation 4	0.95	0.17	3
Fcg2h-EPO BHK preparation 5	0.43	0.18	2

Fcg2h(FN>AQ)-EPO BHK Preparation 1	6.75	2.57	9
Fcg2h(FN>AQ)-EPO BHK Preparation 2	7.38	1.48	4
Fcg2h(FN>AQ)-EPO BHK Preparation 3	7.01	4.64	9
Fcg2h(FN>AQ)-EPO BHK Preparation 4	3.02	0.88	5
Fcg2h(FN>AQ)-EPO BHK Preparation 5	2.77	1.75	5
Fcg2h(FN>AQ)-EPO BHK Preparation 6	5.07	1.64	4
Fcg2h(FN>AQ)-EPO BHK Preparation 7	2.53	0.53	5
Fcg2h(FN>AQ)-EPO BHK Preparation 8	2.92	0.52	5
Fcg2h(FN>AQ)-EPO BHK Preparation 9	1.55	0.66	5
Fcg2h(FN>AQ)-EPO BHK Preparation 10	2.37	1.78	8

Fcg4h-M1-EPO BHK preparation 1	1.09	0.31	7
Fcg4h-M1-EPO BHK preparation 2	1.53	0.35	6

Fcg4h(FN>AQ)-M1-EPO BHK preparation 1	17.16		1
Fcg4h(FN>AQ)-M1-EPO BHK preparation 2	5.87	2.71	7
Fcg4h(FN>AQ)-M1-EPO BHK preparation 3	3.79	0.93	5
Fcg4h(FN>AQ)-M1-EPO BHK preparation 4	4.78	3.42	8

These effects are unexpected and surprising because the effects caused by elimination of the N-linked glycosylation in the IgG2 and IgG4 derived Fc portions are most consistent with reduced on-rate for the erythropoietin receptor. Without wishing to be bound by theory, elimination of the N-linked glycosylation in the IgG2 and IgG4 derived Fc portions may cause an overall conformational change on the Fc-EPO fusion protein.

Example 11. Treatment of beagle dogs with Fc-EPO fusion proteins synthesized in BHK cells

Fc-EPO fusion proteins were administered to beagle dogs to test for effects on hematocrits, reticulocyte counts, and other blood parameters. Specifically, Fcg2h(FN→AQ)-EPO proteins were purified from two independently stably transfected BHK cell lines, clone 65 and clone 187, and administered into beagle dogs intravenously. One male and one female beagle dog were injected with each preparation according to the following schedule:

Day 0: 3 micrograms/kg

Day 16: 10 micrograms/kg

Day 23: 100 micrograms/kg

At various time points after each administration, approximately 2 ml of blood were collected and blood parameters, such as, hematocrits, reticulocyte counts, and other blood parameters, were measured.

The hematocrit responses following treatment are shown in Figure 11. After dosing with 3 mcg/kg of Fc-EPO fusion proteins, blood parameters did not increase from the normal range. Within one week after dosing with 10 mcg/kg, reticulocyte counts increased to over 3% of total blood volume in three of the four animals, and the hematocrits increased to 51 in one animal. Other blood parameters did not increase from the normal range. After dosing with 100 mcg/kg, hematocrit counts rapidly elevated, reaching peak levels of 57 to 62 and remaining above the normal range for five to six weeks. Reticulocyte counts remained elevated for two to three weeks.

For each animal, the number of red blood cells per microliter of blood and the hemoglobin, measured in grams per deciliter, were proportional to the amount of hematocrits. These results indicated that Fc-EPO proteins stimulate the production of red blood cells of normal size with normal hemoglobin content.

Example 12. Purification of Fc-EPO proteins for clinical use

Fc-EPO proteins are purified following standard GMP procedures known to persons skilled in the art. BHK-21 cells, from a banked production clone, are cultured in DMEM/F-12 medium (Invitrogen) supplemented with additional 2.5 mM L-glutamine (Invitrogen), 2 g/l of each HyPep 1501 and HyPep 4601 (Quest International, Chicago, IL), 10 μl/l ethanolamine (Sigma), and 5 μM Tropolone (Sigma) for 7-10 days in batch culture while maintaining high cell viability (*e.g.*, above 80%). The conditioned medium is harvested and clarified by normal-flow-filtration, and is loaded onto a pre-equilibrated Protein A Sepharose Fast-Flow column (Pharmacia), which captures the fusion protein based on the affinity of Protein A for the Fc portion. The column is washed extensively with 15 column volumes of sodium phosphate buffer containing 150 mM sodium phosphate and 100 mM NaCl at neutral pH. The bound protein is eluted at low pH with further 15 column volumes of acidic sodium phosphate buffer of pH 2.5 – 3 but also containing 150 mM sodium phosphate and 100 mM NaCl.

For viral inactivation, the pH of the pooled peak fractions is adjusted to pH 3.8 and incubated for a further 30 minutes at room temperature. After 30-minute incubation, the pooled fractions are neutralized and sterile filtered, then applied to a Q-Sepharose Fast-Flow anion exchange column (Pharmacia), which exploits the acidic pI of the Fc-EPO protein as a result of its extensive sialylation to effectively remove potential contaminants co-eluted with Fc-EPO proteins. Specifically, the neutralized fractions are loaded on a Q-Sepharose Fast-Flow anion exchange column (Pharmacia) at pH 5.0 and eluted with a gradient of NaCl solution. The fractions of Fc-EPO are then collected and pooled for subsequent analysis and for further purification process. For example, the high salt strip from the Q-Sepharose column is applied to a reversed phase chromatography column to remove excess NaCl. The diluted eluant from the reversed phase column is further applied to a second Q-Sepharose Fast Flow (Pharmacia, 3 cm X 9 cm) column.

Potential virus particles are then removed from the pool by nano-filtration (*e.g.*, Viresolve by Millipore). Optionally, further purification steps, such as a hydroxyapatite column or a phenyl-boronate column (binds cis-diols), can be used. Finally, the purified proteins are concentrated to a desired concentration using ultrafiltration and then diafiltered into a suitable formulation buffer. The material is finally sterile filtered, and dispensed into vials to a pre-determined volume.

Example 13. Stress test to determine the stability of Fc-EPO protein formulations.

Vials containing an exemplary sample Fc-EPO formulation or a reference Fc-EPO formulation are stored at 40° C and 75% relative atmospheric humidity, and for defined storage times (*e.g.*, 0 weeks, 4 weeks, 8 weeks, *etc.*). An aliquot sample is taken from each vial after certain storage time and is analyzed. The samples are assessed visually under direct illumination with a cold light source for cloudiness. The cloudiness is further determined by measuring the absorption at 350 nm and 550 nm. In addition, the condition of the Fc-EPO protein in the samples and the presence of protein degradation products are analyzed by analytical size exclusion chromatography (HPLC-SEC). It is found that a formulation containing 0.5 mg/ml Fc-EPO, 10 mM Citrate pH 6.2, 100mM Glycine, 100 mM NaCl, 0.01% w/v polysorbate 20 had significantly increased stability compared to a reference solution.

Example 14. A phase I study of the Fcg2h(FN>AQ)-M1-EPO fusion protein in humans

A Phase I clinical trial of the Fcg2h(FN>AQ)-M1-EPO fusion protein in humans is performed as follows. Pharmacokinetic parameters are determined essentially as described for Aranesp® by MacDougall *et al.* (1999) J. Am. Soc. Nephrol. 10:2392-2395, the teachings of which are hereby incorporated by reference. The terminal serum half-life of intravenously injected Fcg2h(FN→AQ)-M1-EPO fusion protein (dosed at 1 mcg/kg) in humans is found to be between about 20 and 30 hours. Thus, a dose of 1 mcg/kg, or about 70 mcg in an adult anemic patient, results in an initial serum concentration of about 10 ng/ml. Since the normal human erythropoietin concentration is about 0.04 to 0.25 ng/ml (Cazzola *et al.*, (1998) Blood 91:2139-2145), pharmacologically active levels of the Fc-EPO protein remain in the patient's system for at least 5-10 days.

Example 15. A phase II dose finding and dose scheduling study of the Fcg2h(FN→AQ)-M1-EPO fusion proteins

Multicenter, randomized, sequential dose-escalation studies are initiated to investigate the optimum dose and dose schedule for the Fcg2h(FN>AQ)-M1-EPO fusion protein when administered by subcutaneous or intravenous injection in patients with chronic renal failure (CRF) receiving dialysis.

In clinical practice, it is generally convenient to tailor the administration of the Fcg2h(FN→AQ)-M1-EPO fusion protein to an individual anemic patient according to the following guidelines. An initial dose is administered and blood parameters such as the hematocrit, hemoglobin, reticulocyte counts, and platelet counts are monitored. The initial dose is typically between about 0.3 and 3 mcg/kg. A convenient initial dose is 1 mcg/kg. If the increase in hematocrit is less than 5 to 6 per cent of blood volume after 8 weeks of therapy, the dose should be increased. If the increase in hematocrit is greater than 4 per cent of blood volume in a 2-week period, or if the hematocrit is approaching 36%, the dose should be reduced.

An exemplary dosing schedule is as follows.

Once per week dosing: 0.075, 0.225, 0.45, 0.75, 1.5 and 4.5 mcg/kg/dose.

Once per two week dosing: 0.075, 0.225, 0.45, 0.75, 1.5 and 4.5 mcg/kg/dose.

Once per month dosing: 0.45, 0.75, 1.5 and 4.5 mcg/kg/dose.

The studies are carried out in two parts. The first part is a dose-escalation study designed to evaluate the dose of the Fcg2h(FN→AQ)-M1-EPO fusion protein given

either once per week, once per two weeks, or once per month which increases hemoglobin at an optimum rate over four weeks (greater than or equal to 1 g/dL but less than 3 g/dL). The second part of each study is designed to determine the doses required (when administered once per week, once per two weeks, or once per month by either the intravenous or subcutaneous routes of administration) to maintain the hematocrit at the therapeutic target.

Patent Claims

1. A purified dimeric fusion protein, essentially consisting of a dimeric Fc portion of a human IgG molecule comprising a hinge region, a CH2 and a CH3 domain, and human erythropoietin (EPO), wherein each chain of the dimeric Fc portion is linked via its C-terminus directly or via a linker peptide to the N-terminus of an EPO molecule, said fusion protein has the following properties:
(i) the molecule is highly sialylated by comprising 15-28 sialic acid residues;
(ii) the CH2 domain derives from human IgG2 and is modified by replacing the amino acid residues Phe and Asn within the Gln-Phe-Asn-Ser sequence track of the CH2 domain with Ala and Asn, thus forming the sequence Gln-Ala-Gln-Ser within the CH2 domain, and
(iii) the Leu-Ser-Leu-Ser amino acid sequence track near the C-terminus of the CH3 domain is replaced with Ala-Thr-Ala-Thr.

2. A dimeric Fc-EPO fusion protein of claim 1, wherein, additionally, the C-terminal Lys residue of the CH3 domain is replaced with Ala.

3. A dimeric Fc-EPO fusion protein according to claim 1 or 2, wherein the hinge region derives from human IgG1.

4. A dimeric Fc-EPO fusion protein of claim 3, wherein said IgG1 hinge region is modified by replacing the amino acid residue Cys within the Pro-Lys-Ser-Cys-Asp-Lys sequence track of the hinge region with a Ser residue, thus forming the sequence Pro-Lys-Ser-Ser-Asp-Lys within the hinge region.

5. A dimeric Fc-EPO fusion protein according to any of the claims 1 – 4, wherein the erythropoietin portion comprises at least one of the following amino acid substitutions:
(i) a non-cysteine residue at position 29 of the EPO molecule,
(ii) a non-cysteine residue at position 33 of the EPO molecule,
(iii) a cysteine residue at position 88 of the EPO molecule, and
(iv) a cysteine residue at position 139 of the EPO molecule.

6. A dimeric Fc-EPO fusion protein of claim 5, wherein a non-Cys amino acid residue is at position 33 of the EPO molecule instead of the original Cys residue, and a Cys residue is at position 88 of the EPO molecule instead of the original Trp residue, thus enabling the EPO portion within the fusion protein to form a $Cys_{29} - Cys_{88}$ disulfide bond.

7. A dimeric Fc-EPO fusion protein of claim 6, wherein the non-Cys amino acid residue at position 33 is Pro.

8. A dimeric Fc-EPO fusion protein according to any of the claims 1 – 7, wherein the EPO portion comprises one or more mutations selected from the group:
   (i) $Arg_{131} \rightarrow Glu_{131}$
   (ii) $Arg_{139} \rightarrow Glu_{139}$
   (iii) $His_{32} \rightarrow Gly_{32}$
   (iv) $Ser_{34} \rightarrow Arg_{34}$
   (v) $Pro_{90} \rightarrow Ala_{90}$.

9. A dimeric Fc-EPO fusion protein according to any of the claims 1 – 8, wherein the linker peptide comprises a glycosylation site.

10. A dimeric Fc-EPO fusion protein of claim 9, wherein the glycosylation site comprises an Asn-Ala-Thr amino acid sequence.

11. A dimeric Fc-EPO fusion protein according to any of the claims 1 – 10, wherein the complete IgG molecule derives from IgG2 and the hinge regions derives from IgG1.

12. A dimeric Fc-EPO fusion protein according to any of the claims 1 – 11, comprising additionally a CH1 domain.

13. A dimeric Fc-EPO fusion protein according to any of the claims 1 – 12, wherin the fusion protein has 20 – 22 sialic acid residues.

14. A dimeric Fc-EPO fusion protein comprising the sequence:
EPKSSDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWY
VDGVEVHNAKTKPREEQAQSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTK
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSATATPGAAPPRLICDSRVLERYLL
EAKEAENITTGCAEHCSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSEAVLRGQA
LLVNSSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPDAASAAPLRTITADTFRKL
FRVYSNFLRGKLKLYTGEACRTGDR (SEQ ID NO:14).

15. A dimeric Fc-EPO fusion protein comprising the sequence:
EPKSSDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWY
VDGVEVHNAKTKPREEQAQSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTK
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSATATPGAAPPRLICDSRVLERYLL
EAKEAENITTGCAEGPSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSEAVLRGQA
LLVNSSQPCEALQLHVDKAVSGLRSLTTLLRALGAQKEAISPPDAASAAPLRTITADTFRKL
FRVYSNFLRGKLKLYTGEACRTGDR (SEQ ID NO:15).

16. A DNA molecule encoding a fusion protein of any of the claims 1 – 15.

17. A pharmaceutical composition suitable for the treatment of hematopoietic disorders of deficiencies in a mammal comprising in an effective amount an Fc-EPO fusion protein as specified in any of the claims 1 – 15, optionally together with a pharmaceutically acceptable carrier, diluent or excipient.

18. A population of purified highly sialylated Fc-EPO fusion proteins suitable for administration to a mammal, the Fc-EPO fusion proteins comprising an Fc portion towards the N-terminus of the Fc-EPO fusion proteins and an erythropoietin portion towards the C-terminus of the Fc-EPO fusion proteins, said population of fusion proteins having an average of 15-28 sialic acid residues per purified Fc-EPO fusion protein and being obtainable by introducing a DNA molecule encoding a respective Fc-EPO fusion protein into a BHK cell, and expressing, isolating and purifying the population of corresponding Fc-EOP fusion proteins, wherein said population has a

longer serum half-life compared to a population of corresponding Fc-EPO fusion proteins synthesized in NS/0, PerC6, or 293 cells.

19. A population of purified Fc-EPO fusion proteins of claim 18, wherein said population of fusion proteins has an average of 20-22 sialic acid residues per purified Fc-EPO fusion protein.

20. A population of purified Fc-EPO fusion proteins according to claim 18 or 19, wherein the BHK cell is adapted for growth in a protein-free medium or in suspension.

21. A method of producing a population of highly sialylated purified recombinant Fc-EPO fusion proteins comprising an Fc portion towards the N-terminus of the Fc-EPO fusion proteins and an erythropoietin portion towards the C-terminus of the Fc-EPO fusion proteins, said method comprising the steps:
(i) constructing a DNA molecule encoding a Fc-EPO fusion protein;
(ii) transforming a BHK cell with said DNA molecule in a protein –free medium or in suspension,
(iii) expressing the population of Fc-fusion proteins encoded by said DNA molecule,
(iv) harvesting, isolating and purifying said population of Fc-EPO fusion proteins.

22. A method of claim 21, wherein said synthesized population of fusion proteins has an average of 15-28 sialic acid residues per purified Fc-EPO fusion protein.

23. A method of claim 22, wherein said synthesized population of fusion proteins has an average of 20-22 sialic acid residues per purified Fc-EPO fusion protein

24. A method of selecting a BHK cell stably maintaining a nucleic acid sequence encoding an Fc-EPO fusion protein comprising an Fc portion and an erythropoietin portion, the method comprising the steps of: (a) introducing into a BHK cell a nucleic acid sequence encoding hygromycin B and a nucleic acid sequence encoding the Fc-EPO fusion protein; and (b) culturing the BHK cell in the presence of hygromycin B.

25. The method of claim 24, wherein the nucleic acid sequence encoding hygromycin B and the nucleic acid sequence encoding the Fc-EPO fusion protein are present in a single DNA molecule.

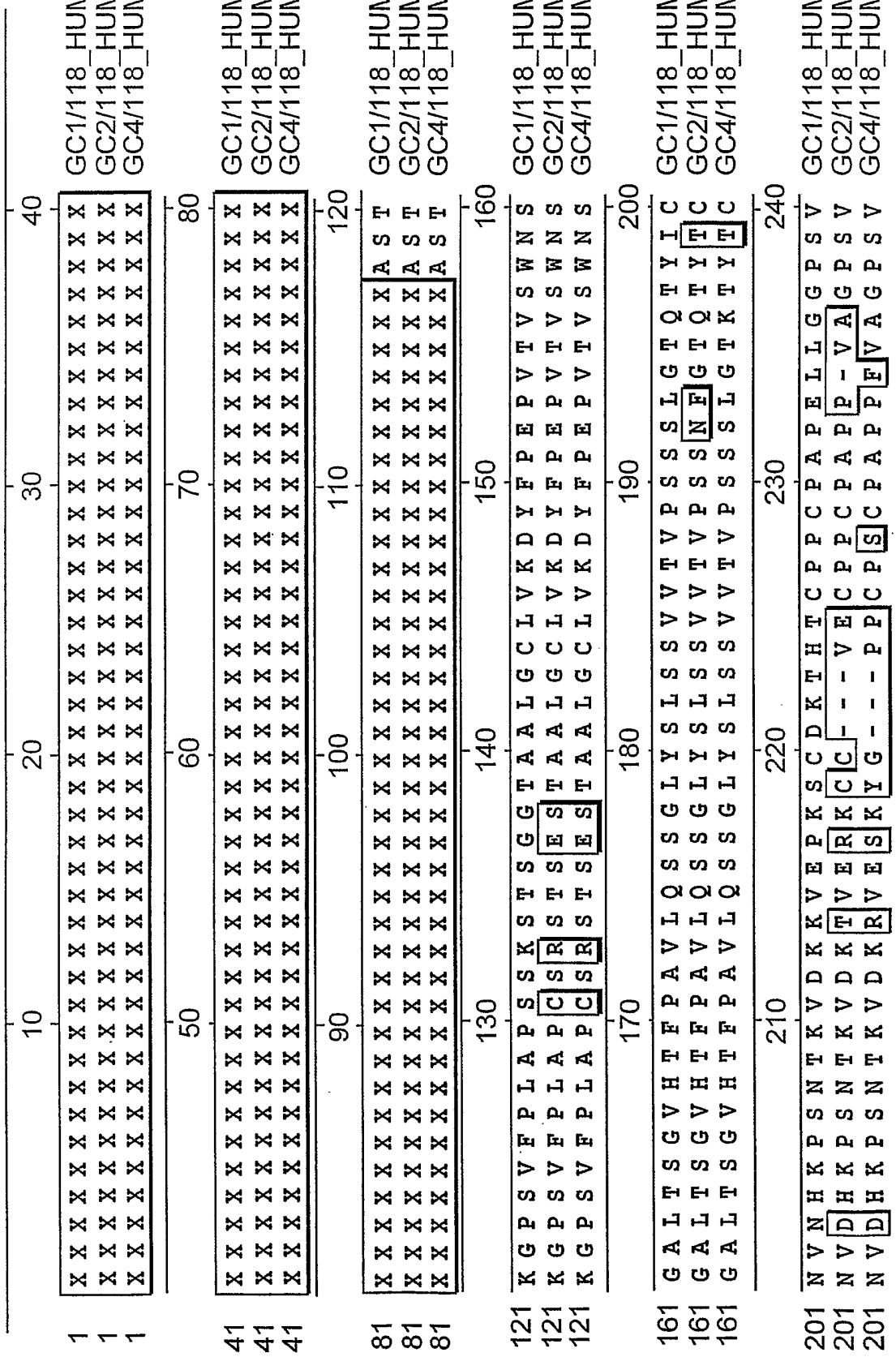

FIG. 1A

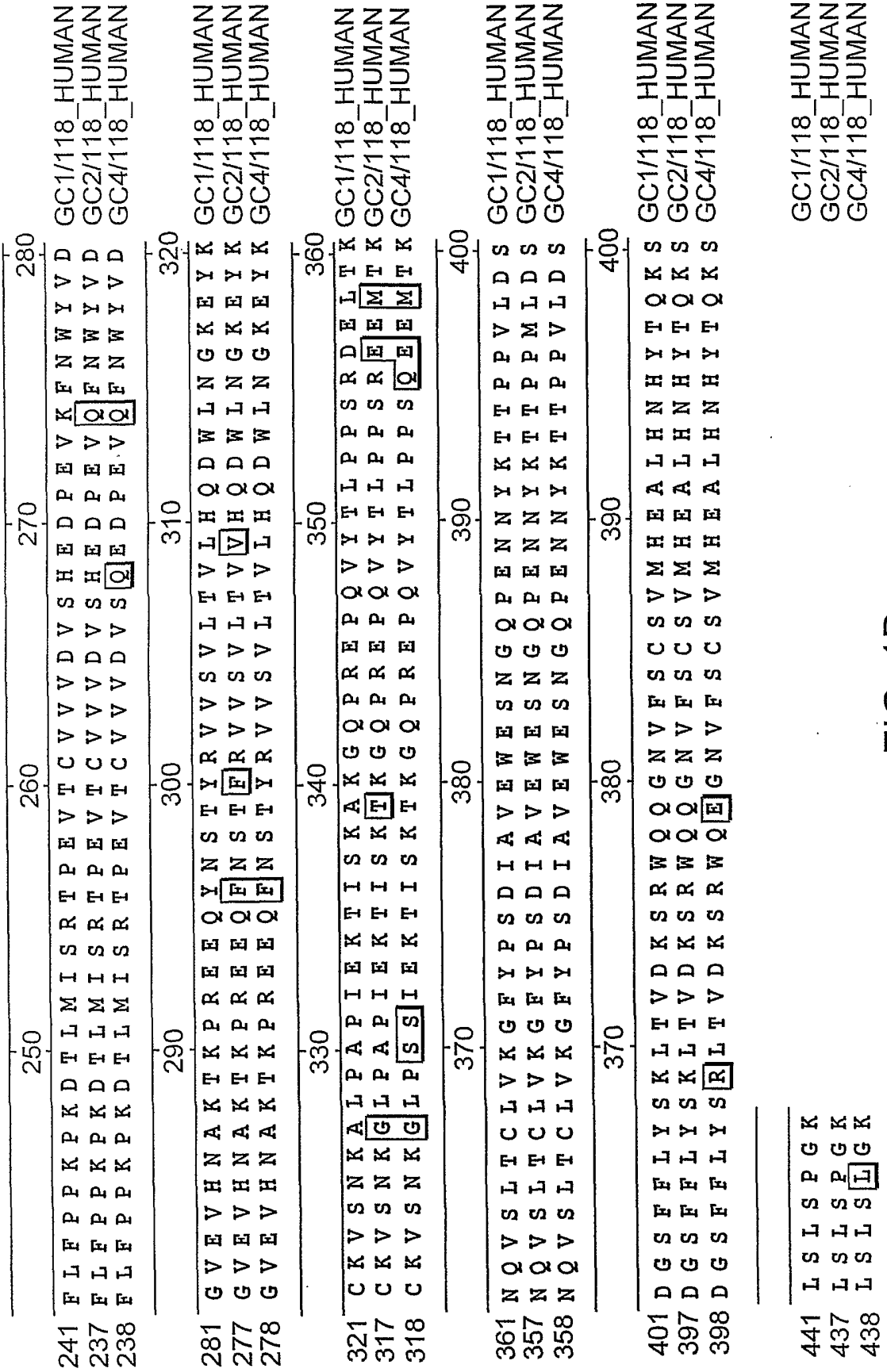
250
260
270
280
290
300
310
320
330
340
350
360
370
380
390
400
GC1/118_HUMAN
GC2/118_HUMAN
GC4/118_HUMAN

FIG. 1B

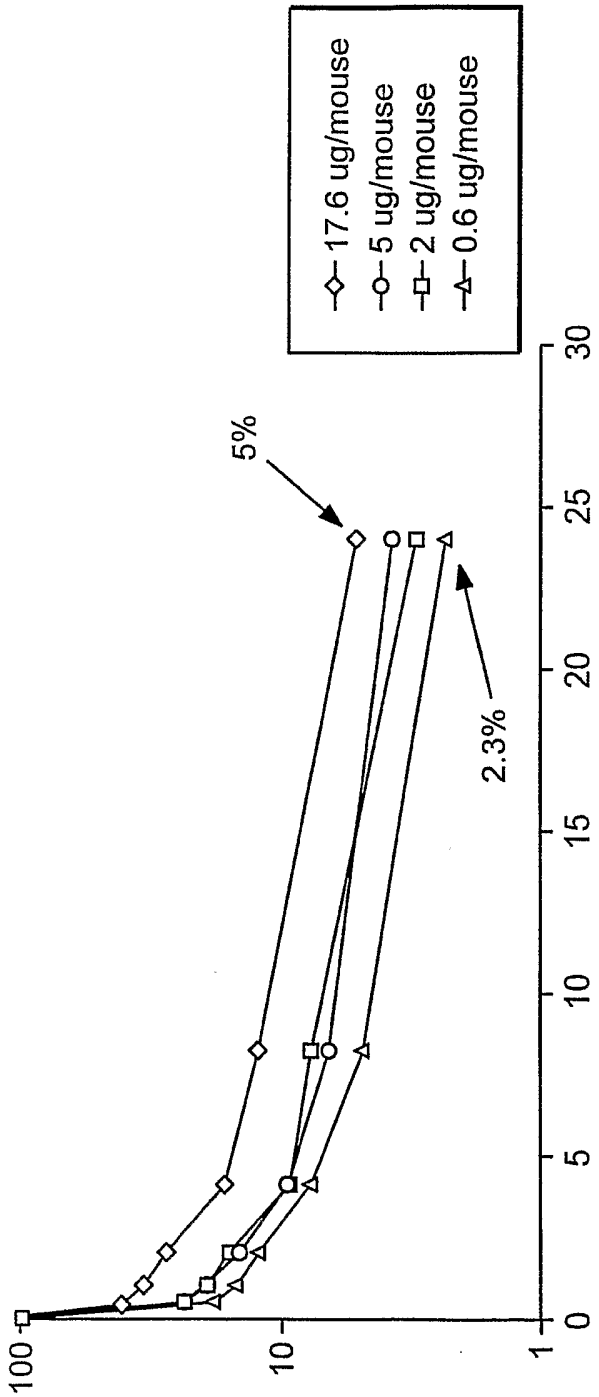
17.6 ug/mouse
5 ug/mouse
2 ug/mouse
0.6 ug/mouse
5%
2.3%
100
10
1
0
5
10
15
20
25
30

FIG. 2

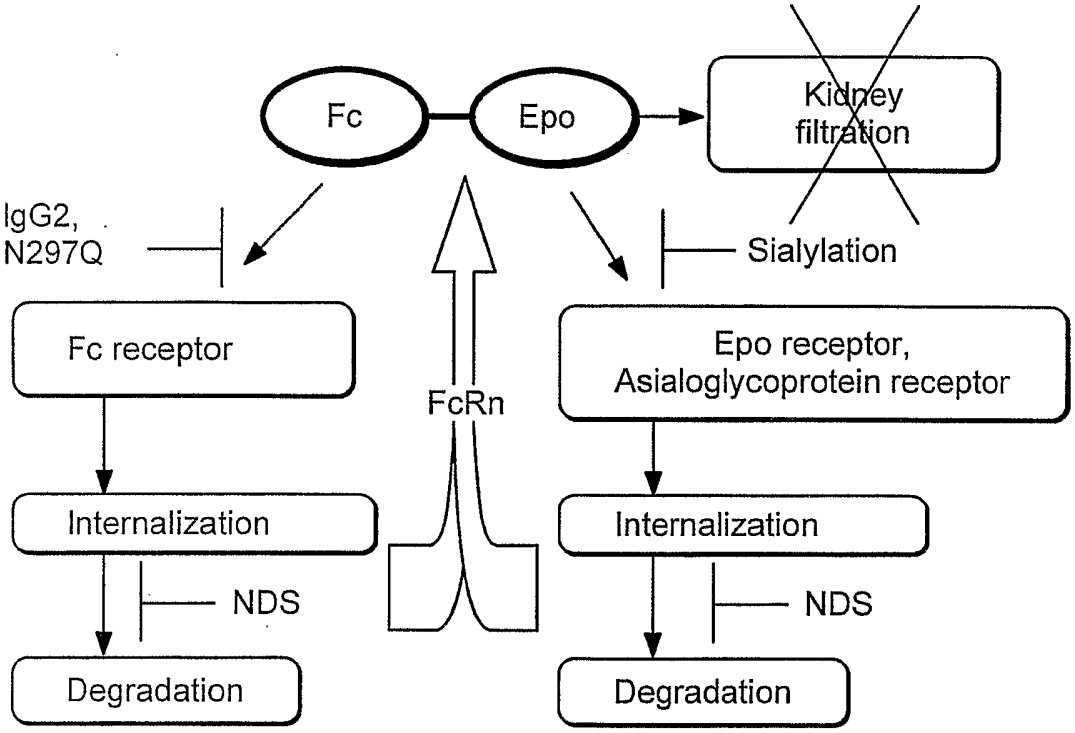
Fc
Epo
Kidney filtration
IgG2, N297Q
Sialylation
Fc receptor
FcRn
Epo receptor, Asialoglycoprotein receptor
Internalization
Internalization
NDS
NDS
Degradation
Degradation

FIG. 3

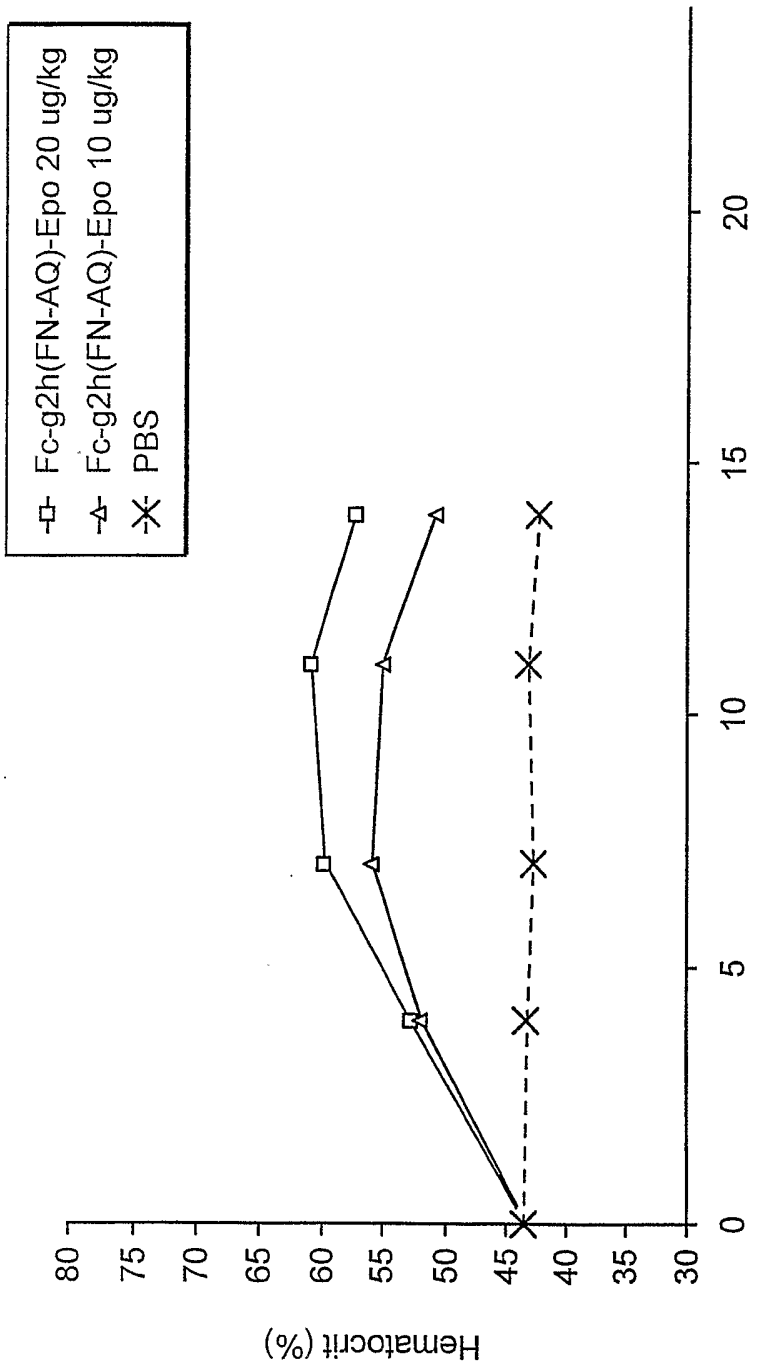
Fc-g2h(FN-AQ)-Epo 20 ug/kg
Fc-g2h(FN-AQ)-Epo 10 ug/kg
PBS
Hematocrit (%)
80
75
70
65
60
55
50
45
40
35
30
0
5
10
15
20
Days

FIG. 4

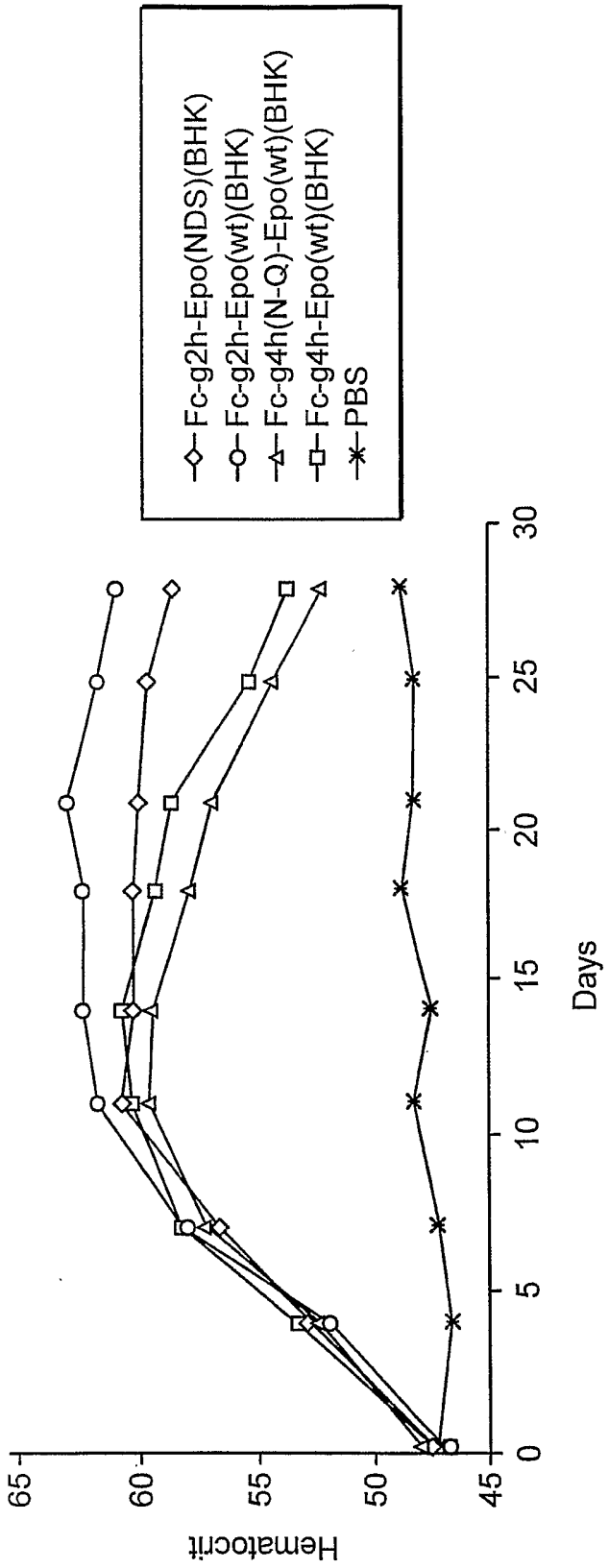
Fc-g2h-Epo(NDS)(BHK)
Fc-g2h-Epo(wt)(BHK)
Fc-g4h(N-Q)-Epo(wt)(BHK)
Fc-g4h-Epo(wt)(BHK)
PBS
Hematocrit
65
60
55
50
45
0
5
10
15
20
25
30
Days

FIG. 5

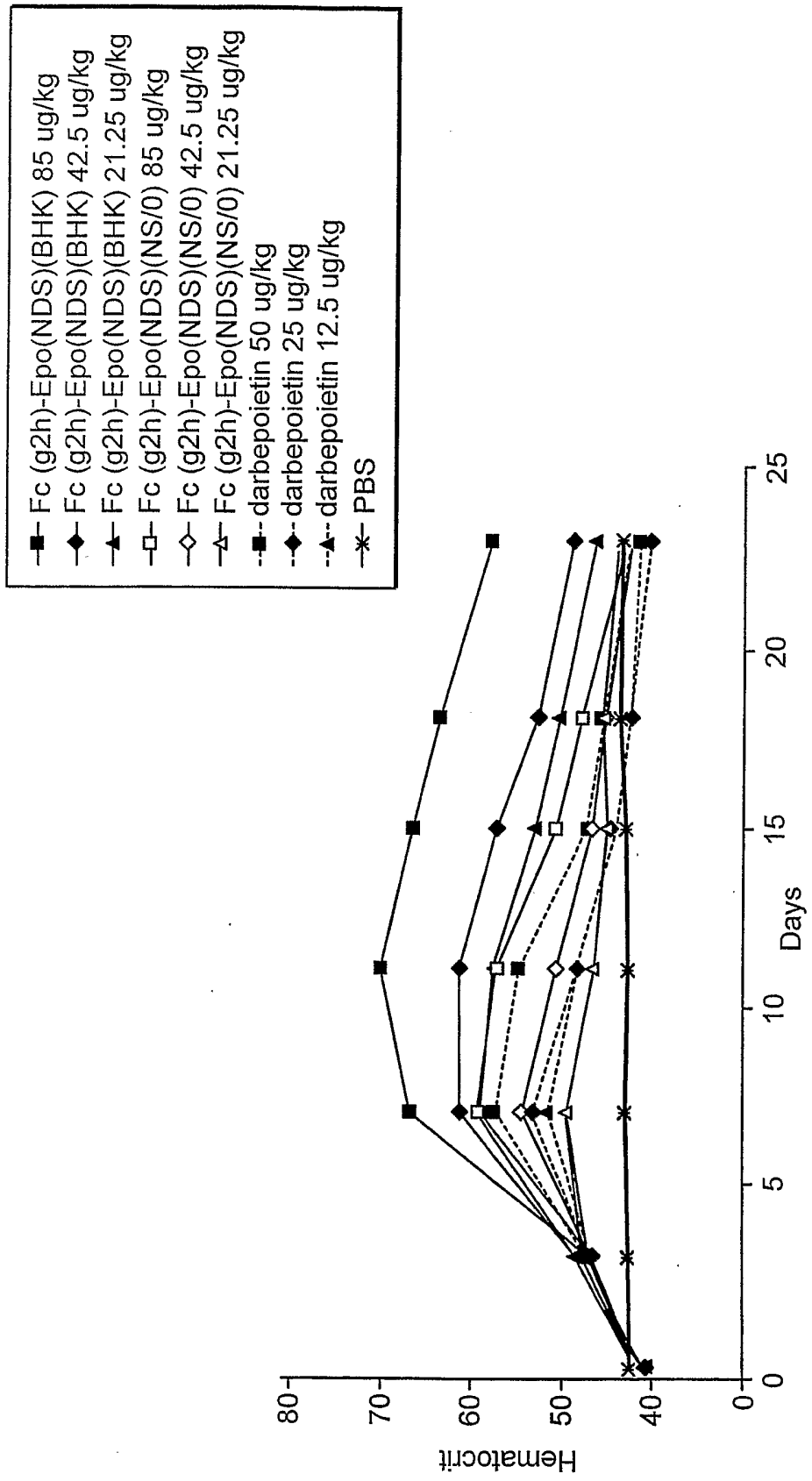
Fc (g2h)-Epo(NDS)(BHK) 85 ug/kg
Fc (g2h)-Epo(NDS)(BHK) 42.5 ug/kg
Fc (g2h)-Epo(NDS)(BHK) 21.25 ug/kg
Fc (g2h)-Epo(NDS)(NS/0) 85 ug/kg
Fc (g2h)-Epo(NDS)(NS/0) 42.5 ug/kg
Fc (g2h)-Epo(NDS)(NS/0) 21.25 ug/kg
darbepoietin 50 ug/kg
darbepoietin 25 ug/kg
darbepoietin 12.5 ug/kg
PBS
Hematocrit
80
70
60
50
40
0
5
10
15
20
25
Days

FIG. 6

Nucleic Acid Sequence encoding mature huFc-EPO:
huFc-g2h(FN>AQ)-M1-EPO

GAGCCCAAATCTTCTGACAAAACTCACACATGCCCACCGTGCCCAGGTAAGCCAGCCCAGGCCTCGCCCTC
CAGCTCAAGGCGGGACAGGTGCCCTAGAGTAGCCTGCATCCAGGGACAGGCCCCAGCTGGGTGCTGACACG
TCCACCTCCATCTCTTCCTCAGCACCACCTGTGGCAGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAA
GGACACCCTCATGATCTCCCGGACCCCTGAGGTCACGTGCGTGGTGGTGGACGTGAGCCACGAAGACCCCG
AGGTCCAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCACGGGAGGAGCAG
GCCCAGAGCACGTTCCGTGTGGTCAGCGTCCTCACCGTTGTGCACCAGGACTGGCTGAACGGCAAGGAGTA
CAAGTGCAAGGTCTCCAACAAAGGCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAACCAAAGGTGGGA
CCCGCGGGGTATGAGGGCCACATGGACAGAGGCCGGCTCGGCCCACCCTCTGCCCTGGGAGTGACCGCTGT
GCCAACCTCTGTCCCTACAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCACGGGAGGAGA
TGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCGTGGAGTGG
GAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACACCTCCCATGCTGGACTCCGACGGCTCCTTCTT
CCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGC
ATGAGGCTCTGCACAACCACTACACGCAGAAGAGCGCCACCGCGACCCCGGGCGCCGCCCCACCACGCCTC
ATCTGTGACAGCCGAGTGCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCGAGAATATCACGACCGGCTG
TGCTGAACACTGCAGCTTGAATGAGAACATCACCGTGCCTGACACCAAAGTGAATTTCTATGCCTGGAAGA
GGATGGAGGTTGGCCAGCAGGCCGTAGAAGTGTGGCAGGGCCTGGCCCTGCTGTCGGAAGCTGTCCTGCGG
GGCCAGGCCCTGTTGGTCAACTCTTCCCAGCCGTGGGAGCCCCTGCAACTGCATGTGGATAAAGCCGTGAG
TGGCCTTCGCAGCCTCACCACTCTGCTTCGGGCTCTGGGAGCCCAGAAGGAAGCCATCTCCCCTCCAGATG
CGGCCTCAGCTGCTCCCCTCCGCACAATCACTGCTGACACTTTCCGCAAACTCTTCCGAGTCTACTCCAAT
TTCCTCCGGGGAAAGCTGAAGCTGTACACAGGGGAGGCCTGCCGGACAGGGGACAGATGA

FIG. 7

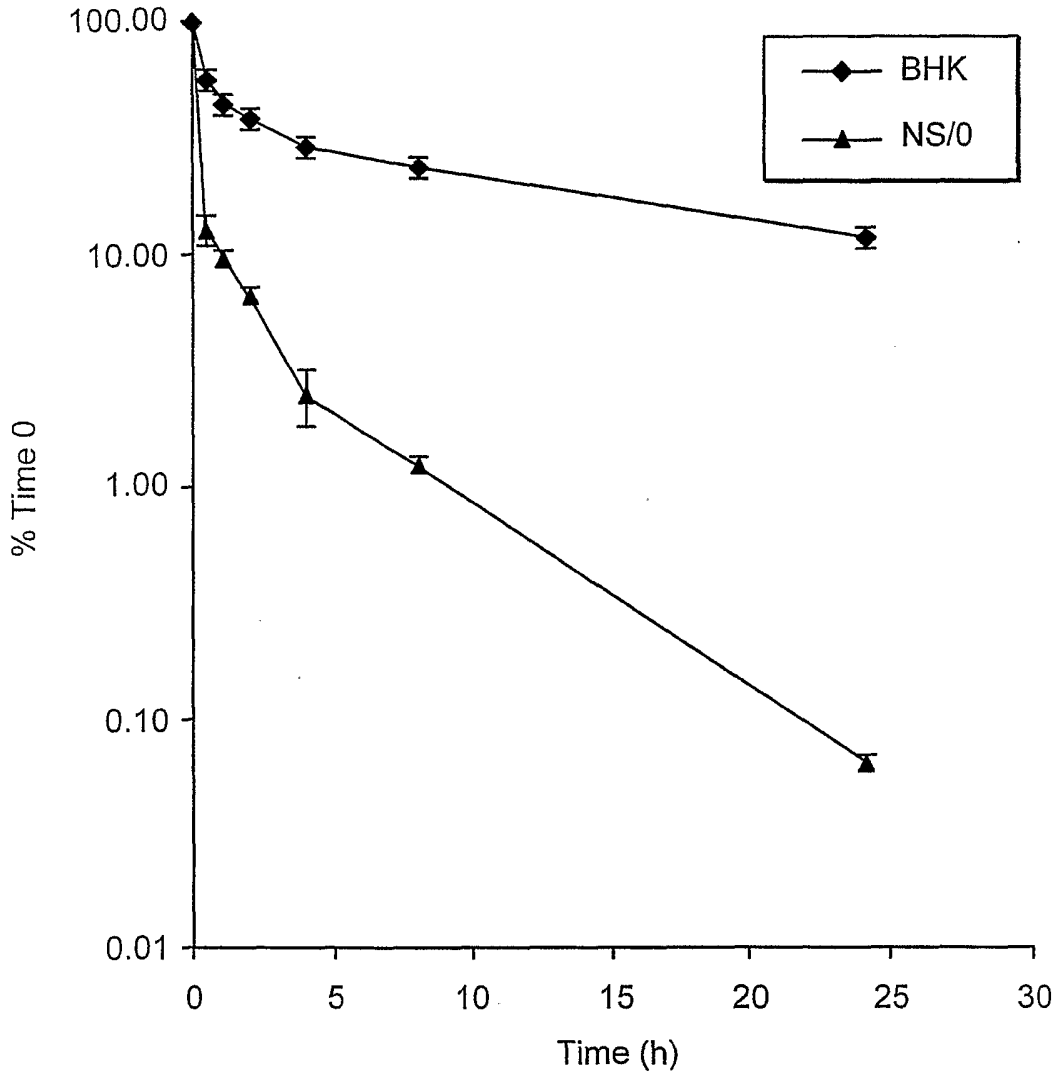

FIG. 8

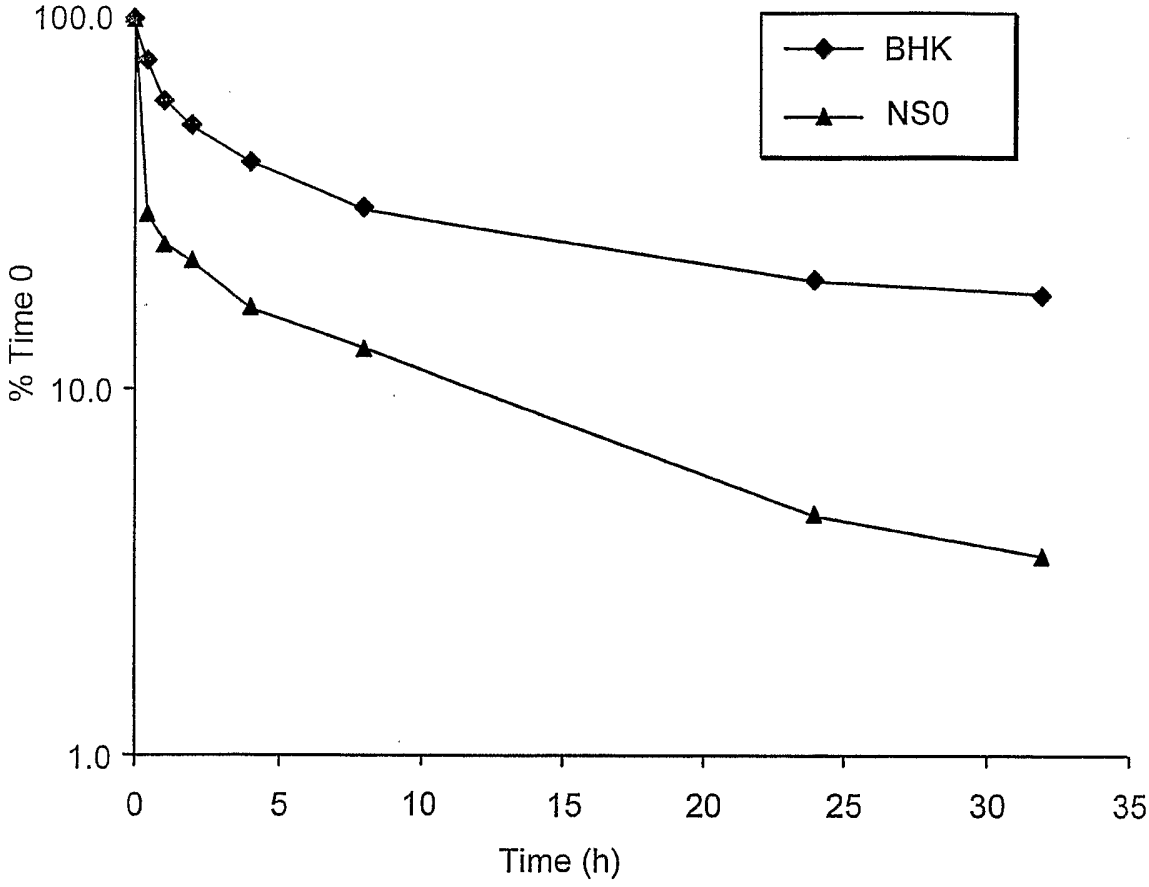

FIG. 9

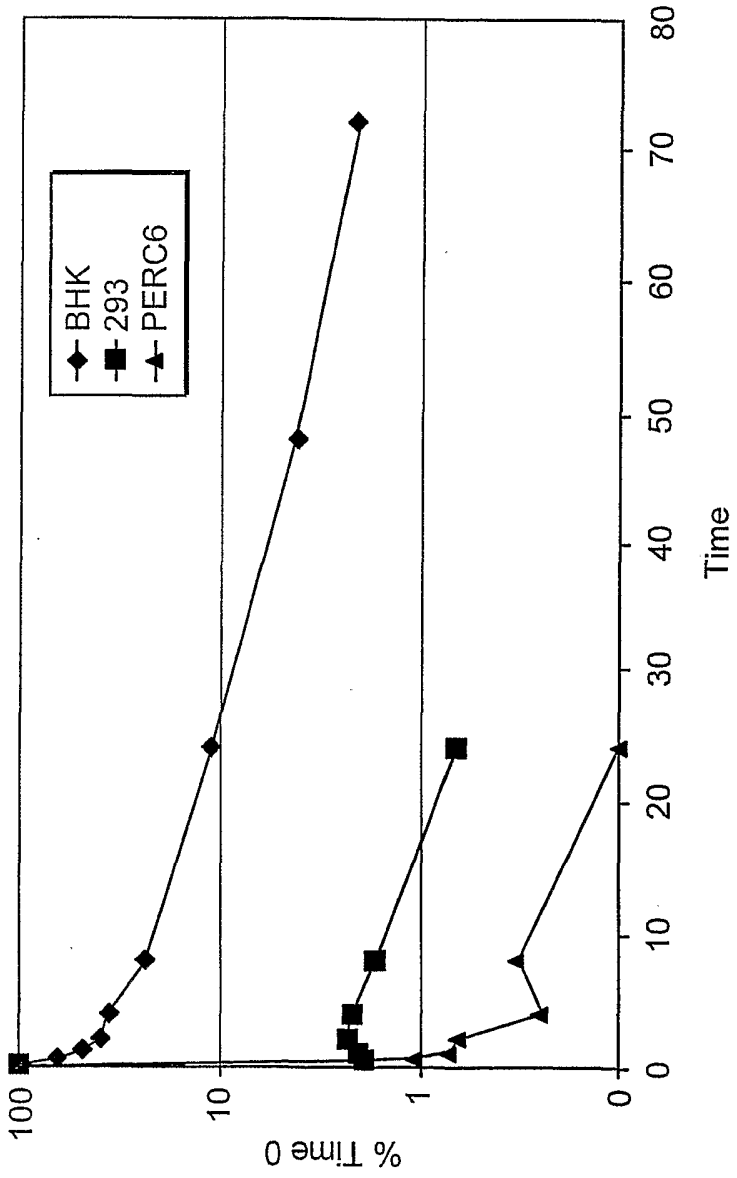

FIG. 10

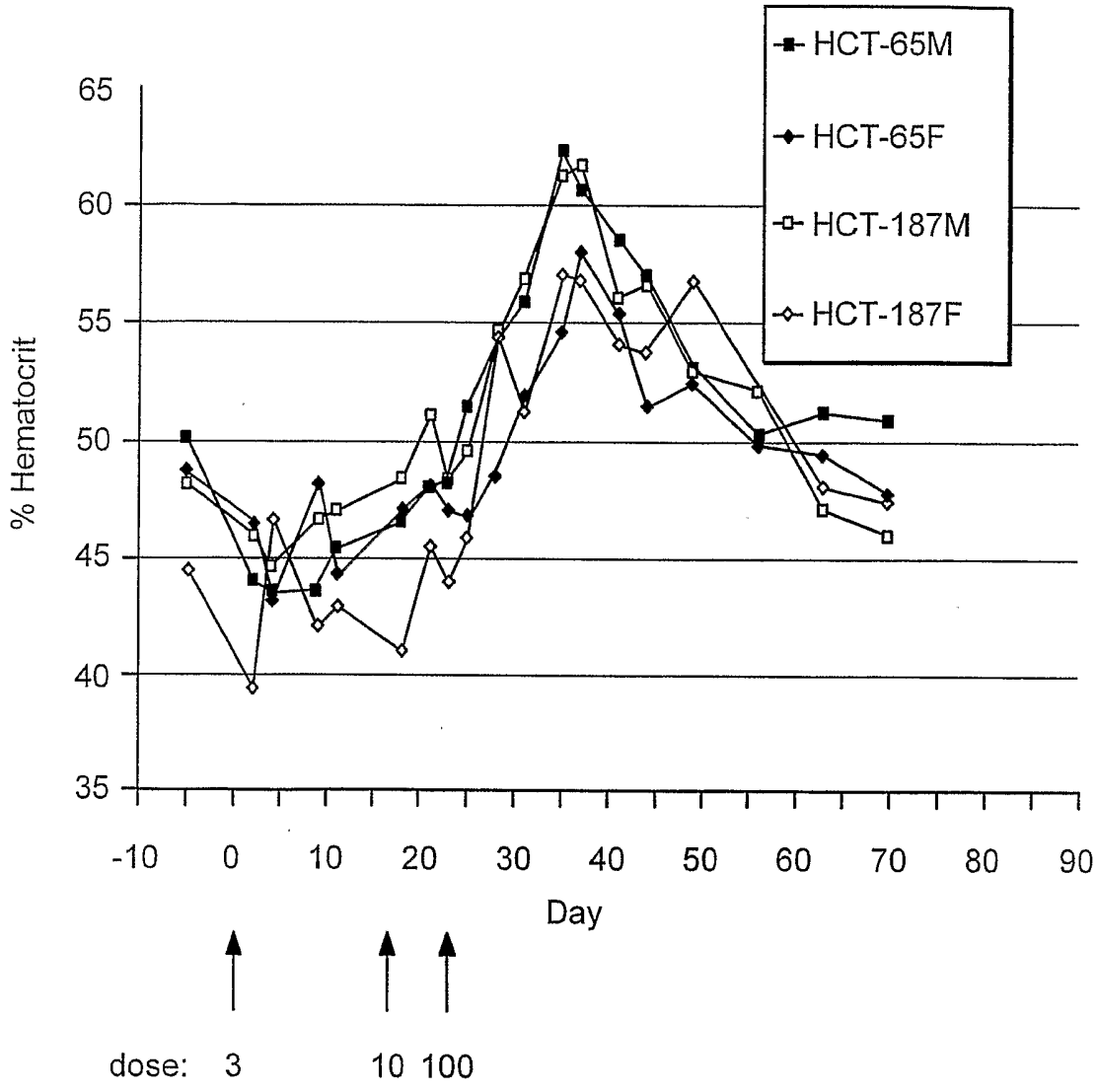
HCT-65M
HCT-65F
HCT-187M
HCT-187F
% Hematocrit
65
60
55
50
45
40
35
-10
0
10
20
30
40
50
60
70
80
90
Day
dose:
3
10
100

FIG. 11

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/014608

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/505

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 01/81405 A (AMGEN INC) 1 November 2001 (2001-11-01) claims 38-42 pp. 20-26 the whole document -----	18-25 1-17
Y	DATABASE UniProt 21 July 1986 (1986-07-21), XP002325441 retrieved from EBI Database accession no. P01859 abstract -----	1-17
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 April 2005	11/05/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Heder, A

Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EGRIE J C ET AL: "DEVELOPMENT AND CHARACTERIZATION OF NOVEL ERYTHROPOIESIS STIMULATING PROTEIN (NESP)" BRITISH JOURNAL OF CANCER, LONDON, GB, vol. 84, no. SUPPL 1, April 2001 (2001-04), pages 3-10, XP001059714 ISSN: 0007-0920 the whole document	1-25
Y	JUNGHANS R P ET AL: "The protection receptor for IgG catabolism is the beta2-microglobulin-containing neonatal intestinal transport receptor." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA. 28 MAY 1996, vol. 93, no. 11, 28 May 1996 (1996-05-28), pages 5512-5516, XP002325439 ISSN: 0027-8424 the whole document	1-25
Y	CHAMOW S M ET AL: "Immunoadhesins: principles and applications" TRENDS IN BIOTECHNOLOGY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 14, no. 2, February 1996 (1996-02), pages 52-60, XP004035817 ISSN: 0167-7799 the whole document	1-25
Y	WO 02/079232 A (LEXIGEN PHARMACEUTICALS CORP) 10 October 2002 (2002-10-10) the whole document	1-25
Y	WO 01/36489 A (MERCK PATENT GMBH) 25 May 2001 (2001-05-25) the whole document	5-8
A	WO 01/58957 A (LEXIGEN PHARMACEUTICALS CORP) 16 August 2001 (2001-08-16) the whole document	
A	LUND J ET AL: "Control of IgG/Fc glycosylation: a comparison of oligosaccharides from chimeric human/mouse and mouse subclass immunoglobulin Gs." MOLECULAR IMMUNOLOGY. JUN 1993, vol. 30, no. 8, June 1993 (1993-06), pages 741-748, XP002325440 ISSN: 0161-5890	

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JEFFERIS R ET AL: "IGG-FC-MEDIATED EFFECTOR FUNCTIONS: MOLECULAR DEFINITION OF INTERACTION SITES FOR EFFECTOR LIGANDS AND THE ROLE OF GLYCOSYLATION" IMMUNOLOGICAL REVIEWS, MUNKSGAARD, vol. 163, June 1998 (1998-06), pages 59-76, XP001203450 ISSN: 0105-2896	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2004/014608

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-17 (entirely), 18-25 (partially)

   Fc-EPO fusion protein comprising 15-28 sialic acid residues wherein the CH2 domain derives from IgG2 and has the Gln-Phe-Asn-Ser sequence motif mutated into Gln-Ala-Gln-Ser, and wherein the CH3 region has the Leu-Ser-Leu-Ser sequence motif mutated into Ala-Thr-Ala-Thr, DNA coding therefor, method of producing said protein, and uses

   ---

2. claims: 18-25 (partially)

   Fc-EPO fusion protein comprising 15-28 sialic acid residues, except the proteins of group 1, DNA coding therefor, method of producing said protein, and uses

   ---

INTERNATIONAL SEARCH REPORT

ɹrmation on patent family members

Inter[redacted]nal Application No
PCT/EP2004/014608

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0181405 A	01-11-2001	AU 5551601 A	07-11-2001
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		EP 1274728 A2	15-01-2003
		JP 2003530874 T	21-10-2003
		MX PA02010333 A	25-04-2003
		WO 0181405 A2	01-11-2001
WO 02079232 A	10-10-2002	BR 0208207 A	28-09-2004
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		MX PA03008715 A	11-12-2003
		WO 02079415 A2	10-10-2002
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		WO 0136489 A2	25-05-2001
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		JP 2003514552 T	22-04-2003
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		CA 2399832 A1	16-08-2001
		CN 1406249 A	26-03-2003
		EP 1252192 A2	30-10-2002
		HU 0204475 A2	28-04-2003
		JP 2003522200 T	22-07-2003
		NO 20023774 A	09-08-2002
		WO 0158957 A2	16-08-2001
		US 2002147311 A1	10-10-2002