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CA 2311684 C 2002/12/10

(11)(21) 2 311 684

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(22) Date de dépôt/Filing Date: 1989/12/22

(41) Mise à la disp. pub./Open to Public Insp.: 1990/06/22

(45) Date de délivrance/Issue Date: 2002/12/10

(62) Demande originale/Original Application: 2 006 596

(30) Priorités/Priorities: 1989/07/31 (199176/89) JP;
1998/12/22 (324747/88) JP

(51) Cl.Int.$^{7}$/Int.Cl.$^{7}$ C07K 14/535, C07K 17/08

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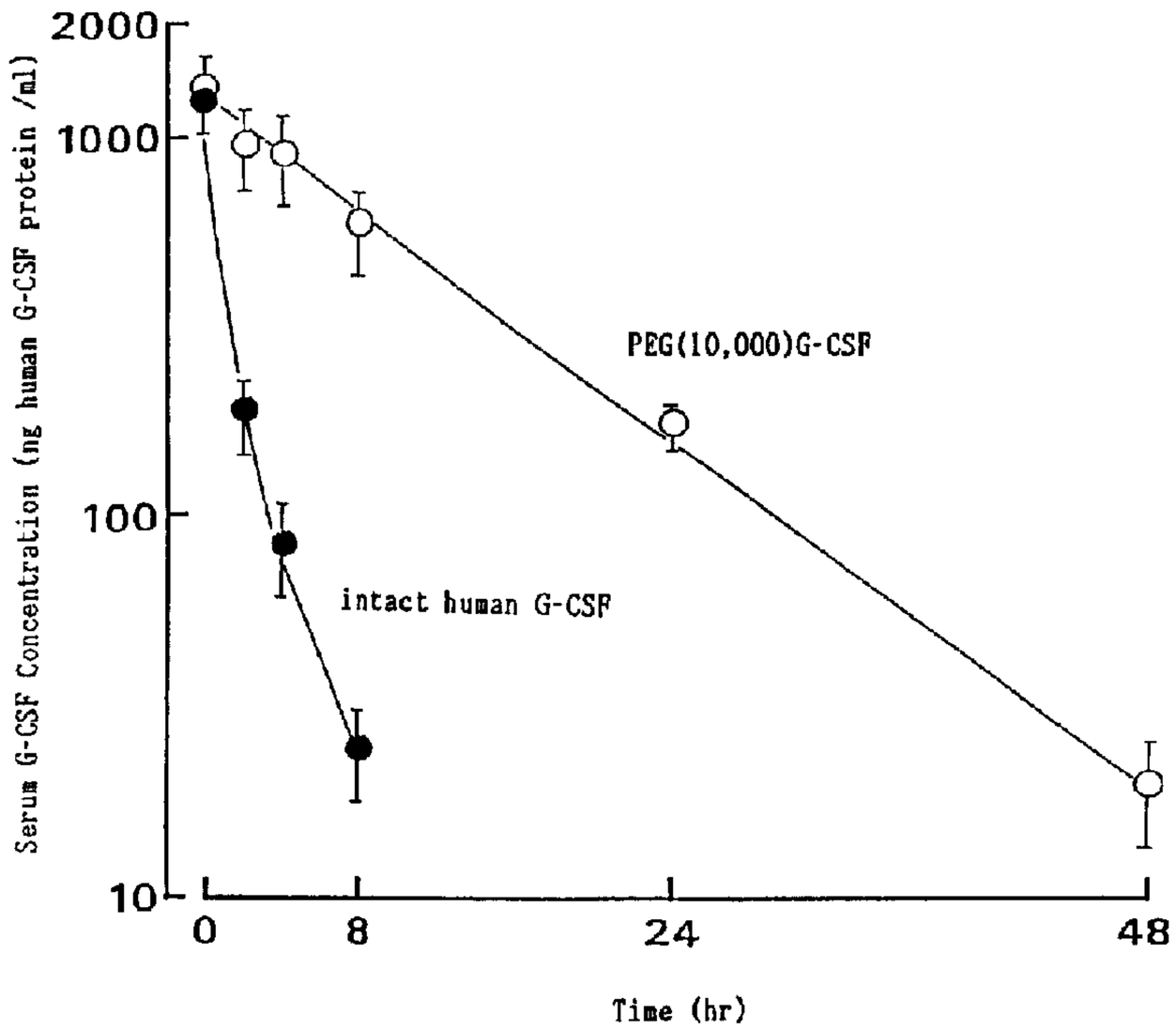
(54) Titre : G-CSF MODIFIE CHIMIQUEMENT
(54) Title: CHEMICALLY-MODIFIED G-CSF




(57) Abrégé/Abstract:
The present invention provides a chemically-modified protein prepared by binding polyethylene glycol to a polypeptide characterized by being the product of expression by a host cell of an exogenous DNA sequence and substantially having the following amino acid sequence: (Het)n Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro (n=0 or 1)
The chemically-modified protein according to the present invention has the prolonged neutrophils-increasing activity and enables a fewer administration with a lower dose.

ABSTRACT:

The present invention provides a chemically-modified protein prepared by binding polyethylene glycol to a polypeptide characterized by being the product of expression by a host cell of an exogenous DNA sequence and substantially having the following amino acid sequence:

(Met)n

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln

Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg

Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu

Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro

Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly

Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser

Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln

Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu

Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu

Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val

Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met

Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro

Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala

Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala

Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr

Arg Val Leu Arg His Leu Ala Gln Pro

(n=0 or 1)

The chemically-modified protein according to the present invention has the prolonged neutrophils-increasing activity and enables a fewer administration with a lower dose.

# CHEMICALLY-MODIFIED G-CSF

## BACKGROUND OF THE INVENTION

The present invention relates to a chemical modification of granulocyte colony-stimulating factor (G-CSF), by which chemical and/or physiological properties of G-CSF can be changed.

Human G-CSF is one of haematopoietic growth factors. It has been shown to be present in the culture medium of a human bladder carcinoma cell line denominated 5637 (ATCC HT8-9) (Welte et al., Proc. Natl. Acad. Sci. (USA), 82, pp.1526-1530, (1985)). The determination of a DNA sequence encoding human G-CSF (Japanese Patent Application Laying Open KOHYO No. 500636/88) has enabled the production of human G-CSF by means of recombinant genetic techniques.

Human G-CSF may be useful in the treatment of haematopoietic disorders including those arising from chemotherapy or from radiation therapy. It may be also useful in bone marrow transplantation. Wound healing burn treatment and the treatment of bacterial inflammation may also benefit from the application of human G-CSF (Welte et al., supra.).

It is generally observed that physiologically-active proteins administered into body can show their pharmacological activity only for a short period of time due to their high clearance rate in body. Furthermore, high hydrophobicity of the proteins would reduce their stability.

For the purpose of decreasing the clearance rate, improving in stability or abolishing antigenicity of the proteins, some methods have been proposed wherein the proteins are chemically modified by using polyethylene glycol. Japanese Patent Application Laying Open KOHYO No. 289522/87 discloses the reduction in immunogenicity of TNF which has been modified by, for EXAMPLE, polyethylene glycol. Japanese Patent Application Laying Open KOHYO No. 503171/87 discloses with respect to IL-2 and IFN-β the reduction in immunogenicity and aggregating property in an aqueous solution, and the prolongation of half-life in blood. In addition, there are disclosed the prolongation of half-life in blood and the disappearance of antigenicity or immunogenicity owing to the modification by polyethylene glycol with respect to a plasminogen activator (Japanese Patent Application Laying Open KOHYO No.60938/88), IL-2, IFN-γ and SOD (Japanese Patent Application Laying Open KOHYO No.10800/88), and IAP (Japanese Patent Application Laying Open KOHYO No.126900/88).

However, these prior arts have not disclosed an improvement in biological activity and pharmacokinetics, which may be expected as a result of the modification of human G-CSF by polyethylene glycol.

Accordingly, it has been desired to prolong the half-life of human G-CSF in body so as to enhance its lasting effect, as may be expected. Furthermore, G-CSF which accerates to recover from neutropenia sooner has been desired.

The present inventors have now found that the above desire can be realized by binding polyethylene glycol to human G-CSF.

DETAILED DESCRIPTION

Any purified and isolated human G-CSF which is produced by host cells such as E. coli and animal cells transformed by using recombinant genetic techniques may be used in the present invention.

Among them, the human G-CSF which is produced by the transformed E. coli is particularly preferable. Such human G-CSF may be obtained in large quantities with high purity and homogeneity and substantially has the following amino acid sequence:

(Met)n

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln
Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro
Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly
Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu
Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu
Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val
Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met
Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro
Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala
Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala
Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr
Arg Val Leu Arg His Leu Ala Gln Pro

(n=0 or 1)

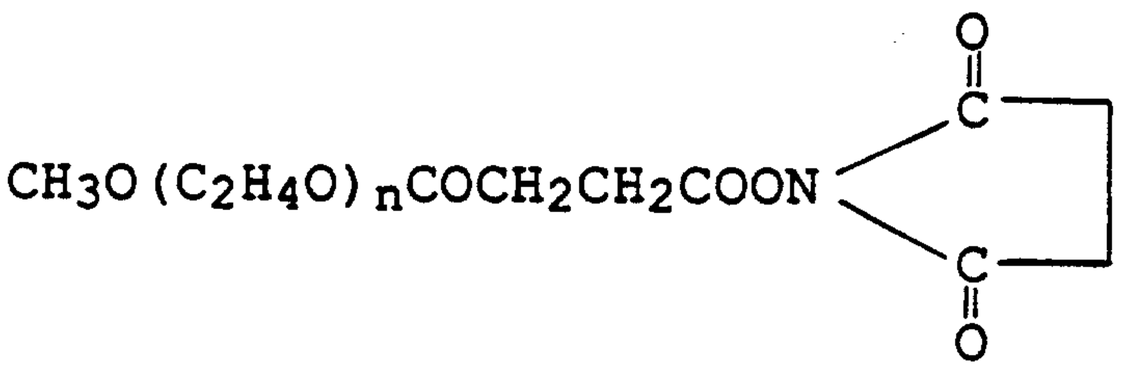
The above human G-CSF may, for EXAMPLE, be prepared according to a method disclosed in Japanese Patent Application Laying Open KOHYO No.500636/88. The wordings "substantially has the following amino acid sequence" mean that the above amino acid sequence may include one or more amino-acid changes (deletion, addition, insertion or replacement) as long as such changes will not cause any disadvantageous non-similarity in function to a naturally-occurring human G-CSF.

It is more preferable to use the human G-CSF substantially having the above amino acid sequence in which at least one lysine, aspartic acid or glutamic acid residue is included.

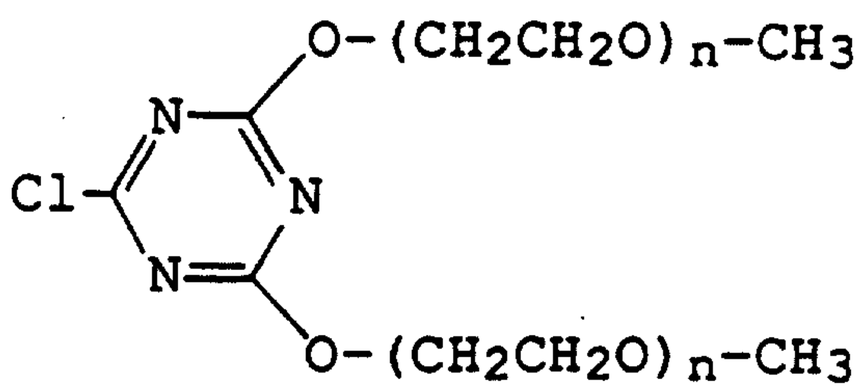
According to the present invention, polyethylene glycol is covalently bound through an amino acid residue of the polypeptide of human G-CSF. The amino acid residue may be any reactive one having, for EXAMPLE, a free amino or carboxyl group, to which a terminal reactive group (a spacer) of polyethylene glycol may be linked. Polyethylene glycol having the spacer is hereinafter referred to as "an activated polyethylene glycol". The amino acid residues having the free amino group may include lysine and N-terminal amino acid residue, and those having the free carboxyl group may include aspartic acid, glutamic acid and C-terminal amino acid residue.

A molecular weight of the polyethylene glycol used in the present invention is not restricted to any particular range, being, however, normally of from 500 - 20,000 and preferably of from 4,000 - 10,000.

Polyethylene glycol is bound onto human G-CSF via its terminal reactive group or the spacer. The spacer, for example, is that which may mediate a bond between the free amino or carboxyl group and polyethylene glycol. The activated polyethylene glycol which may be bound to the free amino group includes Methoxypolyethyleneglycol-Succinimydyl Succinate having the following formula:



which may be prepared by activating succinic acid ester of polyethylene glycol with N-hydroxysuccinylimide. The other activated polyethylene glycol which may be bound to free amino group includes 2,4-bis(O-methoxypolyethyleneglycol)-6-chloro-s-triazine having the following formula:



which had been prepared by reacting polyethylene glycol monomethyl ether with 4-chloro-cyanuric acid. The activated polyethylene glycol which may be bound to the free carboxyl group includes polyoxyethylenediamine having the following formula:

$$H_2NCH_2CH_2CH_2O(C_2H_4O)_nCH_2CH_2CH_2NH_2$$

The chemical modification through a covalent bond may be performed under any suitable condition generally adopted in a reaction of a biologically active substance with activated polyethylene glycol. In case where the reactive amino acid residue in human G-CSF has the free amino group, the above modification is preferably carried out in a buffer solution such as phosphate and borate (pH 7.5 - 10.0) for 1 - 5 hrs at 4 - 37°C. The activated polyethylene glycol may be used in 1 - 200 times, preferably 5 - 50 times the molar amount of the number of free amino group of human G-CSF. On the other hand, in case where the reactive amino acid residue in human G-CSF has the free carboxyl group, the above modification is preferably carried out in pH 3.5 - 5.5, for example, the modification with polyoxyethylenediamine is carried out in the presence of carbodiimide (pH 4.0 - 5.0) for 1 - 24 hrs at 4 - 37°C. The activated polyethylene glycol may be used in 1 - 200 times the molar amount of the number of free carboxyl group of human G-CSF.

The extent of the modification of the amino acid residue may be optionally controlled depending on an amount of the activated polyethylene glycol used in the modification.

A polyethylene glycol-modified human G-CSF, namely chamically modified protein according to the present invention, may be purified from a reaction mixture by a conventional method which is used for purification of proteins, such as dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel chromatography and

electrophoresis . Ion-exchange chromatography is particularly effective in removing unreacted polyethylene glycol and human G-CSF.

The present polyethylene glycol-modified human G-CSF has an enhanced lasting effect, which may be possibly attributed to its prolonged half-life in body.

The present polyethylene glycol-modified human G-CSF has essentially the same biological activity as an intact human G-CSF and may accordingly be used in the same application as that. The polyethylene glycol-modified human G-CSF has an activity for increasing the number of neutrophils, and it is therefore useful in the treatment of haematopoietic disorders including those arising from chemotherapy or from radiation therapy. It may be also useful in the treatment of infection and under receiving the therapy of bone marrow transplantation.

The present polyethylene glycol-modified human G-CSF may be formulated into pharmaceuticals containing also a pharmaceutically acceptable diluent, an agent for preparing an isotonic solution, a pH-conditioner and the like in order to administer them into a patient.

The above pharmaceuticals may be administered subcutaneously, intramuscularly, intravenously or orally, depending on a purpose of treatment. A dose may also change on a kind and condition of the disorder of a patient to be treated, being normally between 0.1 μg and 5 mg by injection and between 0.1 mg and 5 g in an oral administration.

The present invention will be further illustrated by referring to the following EXAMPLES which, however, are not be construed as limiting the scope of the present invention.

EXAMPLE 1

Preparation of PEG (4,500) G-CSF

Recombinant human G-CSF (Japanese Patent Application Laying Open KOHYO No. 500636/88) having the following amino acid sequence was used for the chemical modification according to the present invention:

(Met)n

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln

Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg

Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu

Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro

Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly

Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser

Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln

Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu

Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu

Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val

Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met

Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro

Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala

Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala

Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr

Arg Val Leu Arg His Leu Ala Gln Pro

(n=0. or 1)

As the activated polyethylene glycol (PEG) was used Methoxypolyethyleneglycol-Succinimydyl Succinate (Nippon Oil and Fats, Co., Ltd.) which had been prepared by activating a succinic acid ester of polyethylene glycol with an average molecular weight of about 4,500 with N-hydroxysuccinylimide.

The human G-CSF was reacted in 0.25 M sodium borate buffer (pH 8.0) for 1 hr at 4°C with the activated PEG in 1 - 50 times the molar amount of the free amino group in the human G-CSF. The resulting product was subjected to Sephadex™ G25 which had been equilibrated with 10 mM $NH_4HCO_3$ for buffer-exchange, and then to DEAE ion-exchange chromatography so as to separate the PEG-modified human G-CSF from the unreacted PEG and/or human G-CSF. The resultant PEG-modified human G-CSF is hereinafter referred to as "PEG (4,500) G-CSF".

EXAMPLE 2

Characterization of PEG (4,500) G-CSF

PEG (4,500) G-CSF prepared in EXAMPLE 1 was characterized by the number of unmodified amino groups and a molecular weight estimated by SDS-PAGE.

The number of the unmodified amino groups was determined by reacting them with 0.1% TNBS in 4% $NaHCO_3$ followed by measurement of absorbance at 335 nm (Habeeb et al., Anal. Biochem., 14, pp.328-336, (1966)).

The molecular weight of PEG (4,500) G-CSF was determined by SDS-PAGE (16% gel, CBB staining) according to a method of Laemli, Nature, 227, p.680, 1970. Each lane on

the gel was scanned after staining by using a chromato-scanner (SHIMADZU CORPORATION: CS-930).

When a molar ratio of the activated PEG to the number of free amino group of human G-CSF was increased, the extent of the modification was also increased. The product prepared in said molar ratio of 1 has in addition to a band corresponding to an intact human G-CSF (19 K) another band with an apparent molecular weight of about 26 K (FIG. 1). With respect to the product prepared in the molar ratio of 5 or more, a band with a higher molecular weight was observed besides the above two bands. By scanning the resulting gel, a content of each band was determined. From the result in TABLE 1, it is estimated that the band of 26 K consists of human G-CSF wherein one human G-CSF molecule is coupled with one activated PEG molecule and that a band of 34 K consists of human G-SCF wherein one human G-CSF molecule is coupled with two activated PEG molecules.

TABLE 1

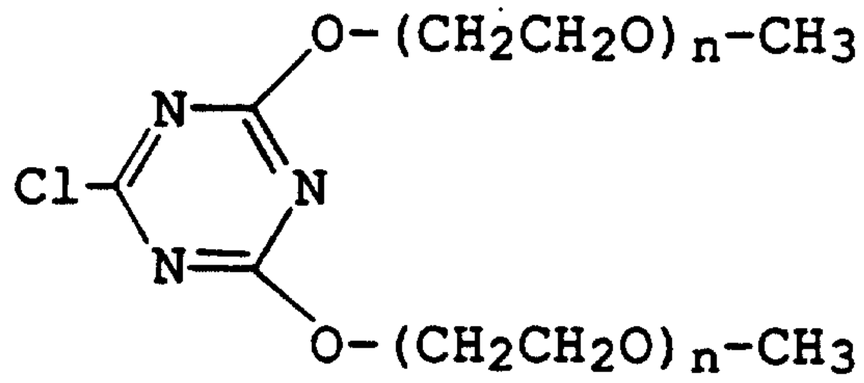
| $PEG/NH_2$ | Distribution | | | Modified $NH_2$ | Unmodified $NH_2$ |
|---|---|---|---|---|---|
| | 19K | 26K | 34K | (%) | (an average number) |
| 1 | 12 | | | 5 | 4.8 |
| 2 | 68 | 31 | 1 | 15 | 4.3 |
| 3 | 56 | 42 | 2 | 15 | 4.3 |
| 4 | 36 | 48 | 16 | 20 | 4.0 |
| 5 | 31 | 49 | 20 | 27 | 3.7 |
| 6 | 25 | 50 | 25 | 27 | 3.7 |
| 7 | 20 | 50 | 28 | 27 | 3.7 |

It was found that based on patterns obtained by SDS-PAGE of the fractions from the ion-exchange chromatography (shown in FIG.1) that the human G-CSF with a higher modification extent was eluted faster from a column and that the fraction finally eluted therefrom contained the unmodified human G-CSF.

EXAMPLE 3

Preparation of PEG (10,000) G-CSF

The same human G-CSF as used in EXAMPLE 1 was modified by an activated polyethylene glycol (Seikagaku Kogyo K.K.) with a molecular weight of about 10,000 having the following formula:




which had been prepared by reacting polyethylene glycol monomethyl ether with 4-chloro-cyanuric acid.

The human G-CSF was incubated with the activated PEG in 5 times the molar amount of the free amino group of the human G-CSF in 0.25 M sodium borate buffer solution (pH 10.0) for 1 hr at room temperature. The resulting product was subjected to Sephadex G25 which had been equilibrated with 10 mM $NH_4HCO_3$ for buffer-exchange, and then to DEAE ion-exchange chromatography to separate the PEG-modified human G-CSF from the unreacted PEG and/or human G-CSF. The resultant PEG-modified human G-CSF is hereinafter referred

to as "PEG (10,000) G-CSF". The estimation of a molecular weight of the product by SDS-PAGE as in EXAMPLE 2 has revealed that its average molecular weight is about 45 K with distributed among 30 K (10%), 40 K (70%) and 66 K (20%). Moreover, the human G-CSF was incubated with the activated polyethylene glycol in 10 times the molar amount of the number of free amino group of the human G-CSF in 0.25 M sodium borate buffer solution (pH 10.0) for 2 hr at room temperature. The resulting product was separated in the same manner as stated above.

It is estimated in the same manner as in EXAMPLE 2 that the product of 30 K consists of human G-CSF wherein one human G-CSF molecule is coupled with one activated PEG molecule.

Furthermore, the human G-CSF was incubated with the activated polyethylene glycol in 50 times the molar amount of the number of free amino group of the human G-CSF.

The estimation of a molecular weight of the resulting products by SDS-PAGE as in EXAMPLE 2 has revealed that its average molecular weight is about 51 K with distributed among 40 K (58 %) and 66 K (42 %).

EXAMPLE 4

Preparation of PEG (4,000) G-CSF

PEG-modified human G-CSF was prepared by covalently binding an activated polyethylene glycol, or polyoxyethylenediamine with an average molecular weight of 4,000 (Nippon Oil and Fats Co., Ltd.) to the above human G-CSF through the free carboxyl group thereof.

The human G-CSF and the activated polyethylene glycol in 60 times the molar amount of the free carboxyl group of the human G-CSF were incubated in the presence of 0.05 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at a room temperature for overnight. The reaction was terminated by adding 1 M sodium acetate (pH 4.75) and further incubated at 25°C in the presence of 0.5 M hydroxyamine for 5 hrs in order to regenerate tyrosine residues. The resulting product was subjected to gel chromatography on TSK G3000SW which had been equilibrated with 10 mM sodium acetate (pH 5.5) to separate the PEG-modified human G-CSF from the unreacted PEG and/or human G-CSF. The resultant PEG-modified human G-CSF is hereinafter referred to as "PEG (4,000) G-CSF". The estimation of a molecular weight of the product by SDS-PAGE as in EXAMPLE 2 has revealed that its molecular weight is distributed among 27 K (70%), 35 K (20%) and 42 K (10%).

EXAMPLE 5

*In vivo* biological assay of PEG (4,500) G-CSF

Male ICR mice (Experiment I: 4 weeks old, Experiment II: 8 weeks old) were used for *in vivo* assays for pharmacological activity of PEG (4,500) G-CSF obtained in EXAMPLE 1. Samples of the intact human G-CSF and PEG (4,500) G-CSF were intravenously injected into mice at a dose of 10 μg or 100 μg protein/kg. At 24 hrs (10 μg protein/kg) or 32 hrs (100 μg protein/kg) after the injection, blood was collected from orbital vein and leukocytes and neutrophils were counted by an auto blood cell counter E-2000 (Toa Medical Electronics, Japan). At

the same time, blood smear was subjected to Wright-Giemsa stain and leukocytes fraction was determined by an auto blood cell analyzer MICROX™ (OMRON TATEISI ELECTRONICS CO.). The results are summarized in TABLE 2 below.

TABLE 2 Effect of PEG-G-CSF on neutrophils in mice

| Group | N | neutrophils ($\times 10^2/\mu l$) | Ratio (to vehicle) |
|---|---|---|---|
| a. 10 $\mu$g/kg | | | |
| < Exp. I > | | | |
| vehicle | 5 | 5.6 ± 1.0 | 1.0 |
| control G-CSF | 6 | 9.6 ± 1.4 | 1.7 |
| PEG(4500) G-CSF(1) | 6 | 20.8 ± 2.6 | 3.7 |
| PEG(4500) G-CSF(2) | 6 | 17.5 ± 3.0 | 3.1 |
| < Exp. II > | | | |
| vehicle | 6 | 12.3 ± 1.7 | 1.0 |
| control G-CSF | 6 | 27.1 ± 4.6 | 2.2 |
| PEG(4500) G-CSF(3) | 6 | 54.0 ± 7.2 | 4.4 |
| b. 100 $\mu$g/kg | | | |
| < Exp. I > | | | |
| vehicle | 6 | 6.6 ± 0.7 | 1.0 |
| control G-CSF | 6 | 18.5 ± 2.3 | 2.8 |
| PEG(4500) G-CSF(1) | 6 | 42.9 ± 4.3 | 6.5 |
| PEG(4500) G-CSF(2) | 6 | 22.6 ± 1.9 | 3.4 |

In TABLE 2, PEG (4,500) G-CSF (1) is a product obtained in the reaction wherein the molar ratio of the activated PEG / the free amino group was five (FIG.1, C), PEG (4,500) G-CSF (2) is a 26 K fraction obtained from DEAE ion-exchange chromatography, and PEG (4,500) G-CSF (3) is a high molecular fraction (26 K:14%, 34 K:55%, >34 K:28%) obtained from said DEAE ion-exchange chromatography.

From the above results, it is observed that the number of neutrophils in the mice injected with PEG (4,500) G-CSFs have been much more increased than those in the mice injected with the intact G-CSF. Especially, PEG (4,500) G-CSFs (1) and (3) with a higher extent of the modification showed a remarkable increase in the number of neutrophils.

When human G-CSF is injected into mice at a dose of 10 μg protein/kg, the number of neutrophils increases, and generally at 6 - 12 hrs after the injection, it gets to the maximum. After that, the number of neutrophils decreases slowly to a basal level about 30 hrs after injection. When 10 μg protein/kg injection, 24 hrs corresponds to the time span as normally required for the number of neutrophils which has once increased to again decrease almost to a basal level. For this reason, the time for collection of blood was determined. Accordingly, the above result may indicate that the half-life of human G-CSF activity in mice has been prolonged by the present modification.

A simple mixture of human G-CSF and PEG did only show the same result as the intact human G-CSF (Data are not shown).

EXAMPLE 6

*In vivo* biological assay of PEG (4,000) G-CSF

Male ICR mice (7 weeks old) were used for *in vivo* assays for pharmacological activity of PEG (4,000) G-CSF obtained in EXAMPLE 4. Samples of the intact human G-CSF and PEG (4,000) G-CSF were intravenously injected into mice at a dose of 10 μg protein/kg. At 24 hrs after the injection, blood was collected from orbital vein and leukocytes and neutrophils were counted as in EXAMPLE 5. The results are shown in TABLE 3.

TABLE 3

| Group | Number of Animals | Number of Neutrophils ($\times 10^2$ / μl) | Ratio |
|---|---|---|---|
| Vehicle | 6 | 10.9 ± 1.0 | 1.0 |
| G-CSF | 6 | 16.4 ± 1.4 | 1.5 |
| PEG (4,000) G-CSF | 6 | 23.3 ± 2.5 | 2.1 |

It has been revealed that PEG (4,000) G-CSF in which the activated PEG is bound through the free carboxyl group has increased the number of neutrophils much more than the intact human G-CSF has.

EXAMPLE 7

Effects of PEG-modified human G-CSFs on mice neutrophils

Male ICR mice (7 weeks old) were used for *in vivo* assays for pharmacological activity of PEG (4,500) G-CSF and PEG (10,000) G-CSF obtained in EXAMPLES 1 and 3, respectively. PEG (4,500) G-CSF used here is a high

molecular fraction from DEAE ion-exchange chromatography of a product obtained in the reaction wherein the molar ratio of the activated PEG / the free amino group was fifty (an average molecular weight of 60K; 38K:20%, 58K:54%, 80K:27%). Samples of the human G-CSF, PEG (4,500) G-CSF and PEG (10,000) G-CSF were intravenously injected into mice at a dose of 10 μg protein/kg. At 6, 24, 32, 48 and 72 hrs after the injection, blood was collected from orbital vein and leukocytes and neutrophils were counted as in EXAMPLE 5, except for using an auto blood cell counter CC180-A (Toa Medical Electronics, Japan). FIG.2 shows the resulting time course of change in the number of neutrophils. A significant neutrophilia was observed over 32 hrs and 48 hrs for PEG (4,500) G-CSF and PEG (10,000) G-CSF, respectively. On the other hand, neutrophilia induced by the intact human G-CSF was maintained for 24 hrs.

Moreover, male ICR mice (8 weeks old) were intravenously administered with the PEG (10,000) G-CSFs obtained in EXAMPLE 3 (30 K, an average molecular weight of 51 K) at a dose of 10 μg protein/kg. At 24 hours after the injection the number of neutrophils was counted as in EXAMPLE 5. The results are shown in TABLE 4.

TABLE 4

| Group | Number of Animals | Number of Neutrophils (x $10^2$ / μl) | Ratio (to vihicle) |
|---|---|---|---|
| Vehicle | 5 | 7.4 ± 0.6 | 1.0 |
| G-CSF | 5 | 16.4 ± 3.1 | 2.2 |
| PEG(10,000)G-CSF 30K | 5 | 68.9 ± 10.5 | 9.3 |
| PEG(10,000)G-CSF 51K | 5 | 95.8 ± 6.4 | 12.9 |

Especially, PEG (10,000) G-CSF having an average molecular weight of 51 K with a higher extent of the modification showed a remarkable increase in the number of neutrophils.

EXAMPLE 8

Effects of PEG-modified human G-CSF on cyclophosphamide-induced neutropenic mice

Male ICR mice (7 weeks old) were intravenously administered with the same PEG (4,500) G-CSF and PEG (10,000) G-CSF as used in EXAMPLE 7 at a dose of 10 μg protein/kg once a day for successive 4 days starting from one day after 200 mg/kg cyclophosphamide intraperitoneal injection. At 6, 24 and 48 hrs after the last injection, blood was collected from orbital vein and leukocytes and neutrophils were counted as in EXAMPLE 5.

As shown in FIG.3, PEG-modified G-CSFs have accelerate the recovery from neutropenia induced by the injection of cyclophosphamide. Especially, PEG (10,000) G-

CSF has effected a significant increase in number of neutrophils.

EXAMPLE 9

Effects of PEG-modified human G-CSF on 5-FU-induced neutropenic mice

Female BDF1 mice (7 weeks old) were subcutaneously administered with the intact human G-CSF and the same PEG (10,000) G-CSF as used in EXAMPLE 7 at a dose of 10 μg protein/kg once a day for successive 11 days (PEG-1), for every other day (at day 1, 3, 5, 7, 9 and 11; PEG-2) and every third day (at day 1, 4, 7 and 10; PEG-3) starting from one day after 200 mg/kg 5-FU intravenous injection. At 7, 8, 9, 10, 11, 12, 14 and 17 days, blood was collected from orbital vein and leukocytes and neutrophils were counted as in EXAMPLE 5.

As shown in FIG.4, peripheral neutrophil counts of mice injected with PEG (10,000) G-CSF has been recovered to a basal level two days sooner than those injected with the intact human G-CSF. Moreover, it is found that PEG-2 and PEG-3 may show almost the same effect as PEG-1 on the recovery from neutropenia induced by the injection of 5-FU, suggesting that PEG (10,000) G-CSF possesses a higher and prolonged neutrophil-recovering activity.

EXAMPLE 10

Acute toxicity of PEG-modified human G-CSF

Male and female ICR mice (5 weeks old) groups consisting 6 mice each were intravenously administered with the same PEG (4,500) G-CSF and PEG (10,000) G-CSF as used in EXAMPLE 7 as well as vehicles at a dose of 12 ml/kg.

General condition and survival of the treated mice were observed as often as possible for 6 hrs immediately after administration and once a day for the following 14 days. The body weight was checked at the day of injection, 5, 8, 12 and 15th days. Surviving mice were bled to death under ether anesthesia and subjected to pathologic autopsy.

As shown in TABLE 5, no mouse administered with PEG-modified human G-CSF did die for the observed period. LD 50 for both PEG (4,500) G-CSF and PEG (10,000) G-CSF was estimated over 3,000 μg protein/kg in both male and female mice. No remarkable change in general condition, body weight or opinion of the autopsy was observed for PEG (4,500) G-CSF or PEG (10,000) G-CSF. These results may suggest that the acute toxicity of PEG-modified human G-CSF is very weak.

**TABLE.5** Mortality of male and female mice

| Sex | Compound | Dose (μg/kg) | Number of deaths on day 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 (Day) | Mortality* | $LD_{50}$ (μg/kg) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Male | Vehicle | — | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0/6 | — |
| | PEG4500-G-CSF | 3,000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0/6 | >3,000 |
| | PEG10000-G-CSF | 3,000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0/6 | >3,000 |
| Female | Vehicle | — | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0/6 | — |
| | PEG4500-G-CSF | 3,000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0/6 | >3,000 |
| | PEG10000-G-CSF | 3,000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0/6 | >3,000 |

‡ : No. of dead animals / No. of treated animals

EXAMPLE 11

Study of pharmacokinetics of PEG (10,000) G-CSF

Male Sprague-Dawley rats (7 weeks old) were used for study of pharmacokinetics of the intact human G-CSF and PEG (10,000) G-CSF prepared in FIG. 3. Samples were intravenously injected into rats at a dose of 100 μg protein/kg. At 10 min, 2, 4, 8, 24 and 48 hrs after the injection, about 6 -7 ml of blood was collected from abdominal aorta into a polypropylene tube of about 15 ml volume and centrifuged (18,000 x g) at 4°C for 5 min to separate a serum fraction. An amount of the active human G-CSFs contained in the serum fraction was determined by a bioassay for proliferation induction of mouse bone marrow cells on the basis of incorporation of $^{3}H$-thymidine (Ralph et al., Blood 66, pp.633-639, (1986)). Average serum concentration data are shown in FIG. 5. Each point represents the mean value of three animals with standard deviation indicated by bars. The results indicate that the half lives of the intact human G-CSF and PEG (10,000) G-CSF are 1.79 hrs and 7.05 hrs, respectively, and AUCs are also 2,000 ng protein hrs/ml and 16,195 ng protein hrs/ ml, respectively. Accordingly, it is demonstrated that the half life of PEG (10,000) G-CSF has been prolonged about 4-fold over that of the intact human G-CSF.

From the above results, it is expected that the present PEG-modified human G-CSF may make a great contribution to the treatment with human G-CSF because it has the prolonged neutrophils-increasing activity, enabling a fewer administration with a lower dose.

## BRIEF DESCRIPTION OF DRAWINGS

FIG.1 shows scanning patterns of PEG (4,500) G-CSF obtained by SDS-PAGE. The molar ratio of the activated PEG to the free amino group is 0 for (a), 1 for (b), 5 for (c), 10 for (d) and 50 for (e), respectively. The peak of the unmodified human G-CSF is marked with *.

FIG.2 shows the time course of the change in number of neutrophils in mice. Each point represents an average value obtained from six mice with a standard deviation.

FIG.3 shows an accelerating effect of PEG-modified human G-CSF on the recovery from neutropenia induced by cyclophosphamide. Each point represents an average value obtained from six mice with a standard deviation.

FIG.4 shows an accelerating effect of PEG-modified G-CSF on the recovery from neutropenia induced by 5-FU. Each point represents an average value obtained from six mice with a standard deviation.

FIG.5 shows the results obtained in the study of pharmacokinetics of PEG (10,000) G-CSF.

**CLAIMS:**

1. A biologically active G-CSF polypeptide having the natural complement of lysine residues and having at least one polyethylene glycol molecule covalently attached to at least one amino acid of the polypeptide.

2. The G-CSF polypeptide according to claim 1 wherein said polyethylene glycol molecules is covalently attached through an amino group of an amino acid of the polypeptide.

3. A biologically active G-CSF polypeptide having at least one polyethylene glycol molecule covalently attached to at least one amino acid of the polypeptide, wherein said G-CSF polypeptide is not a lysine-depleted variant.

4. The G-CSF polypeptide according to claim 3 wherein said polyethylene glycol molecule is attached through an amino group of an amino acid of the polypeptide.

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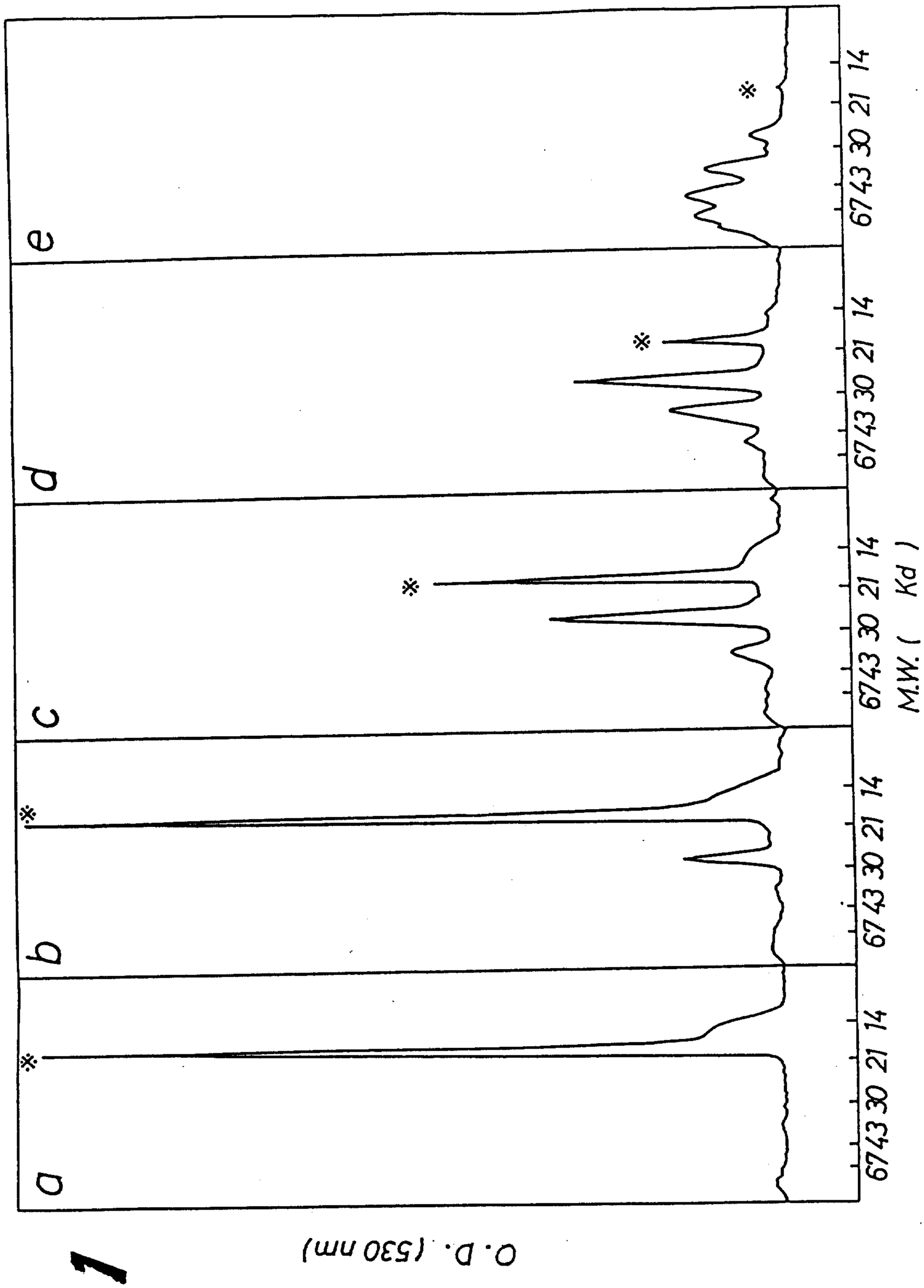



FIG. 1

FIG. 2

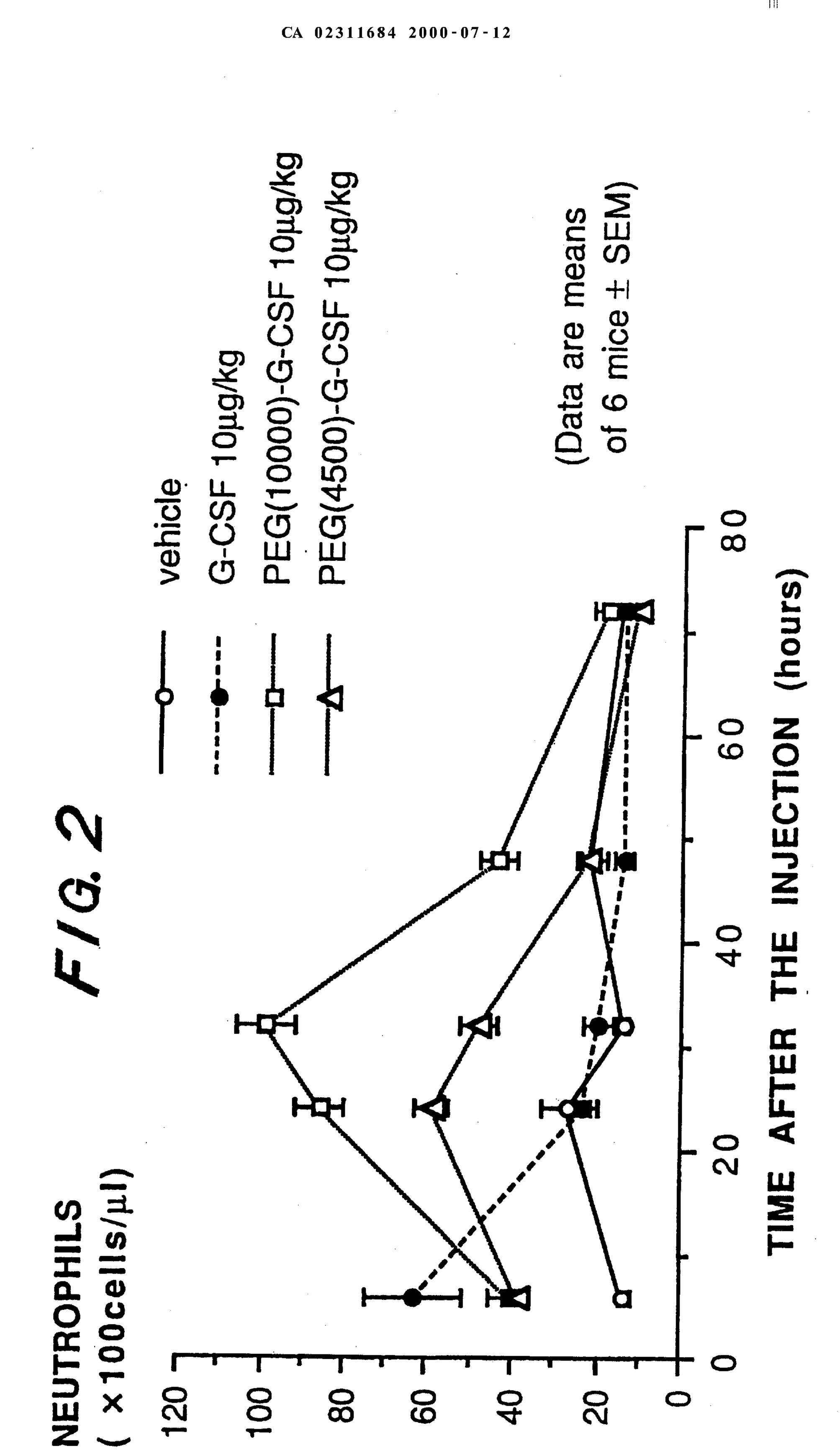

vehicle
G-CSF 10µg/kg
PEG(10000)-G-CSF 10µg/kg
PEG(4500)-G-CSF 10µg/kg
(Datas are means of 6 mice ± SEM)
NEUTROPHILS ( x100cells/µl)
120
100
80
60
40
20
0
0
20
40
60
80
TIME AFTER THE INJECTION (hours)

FIG. 3

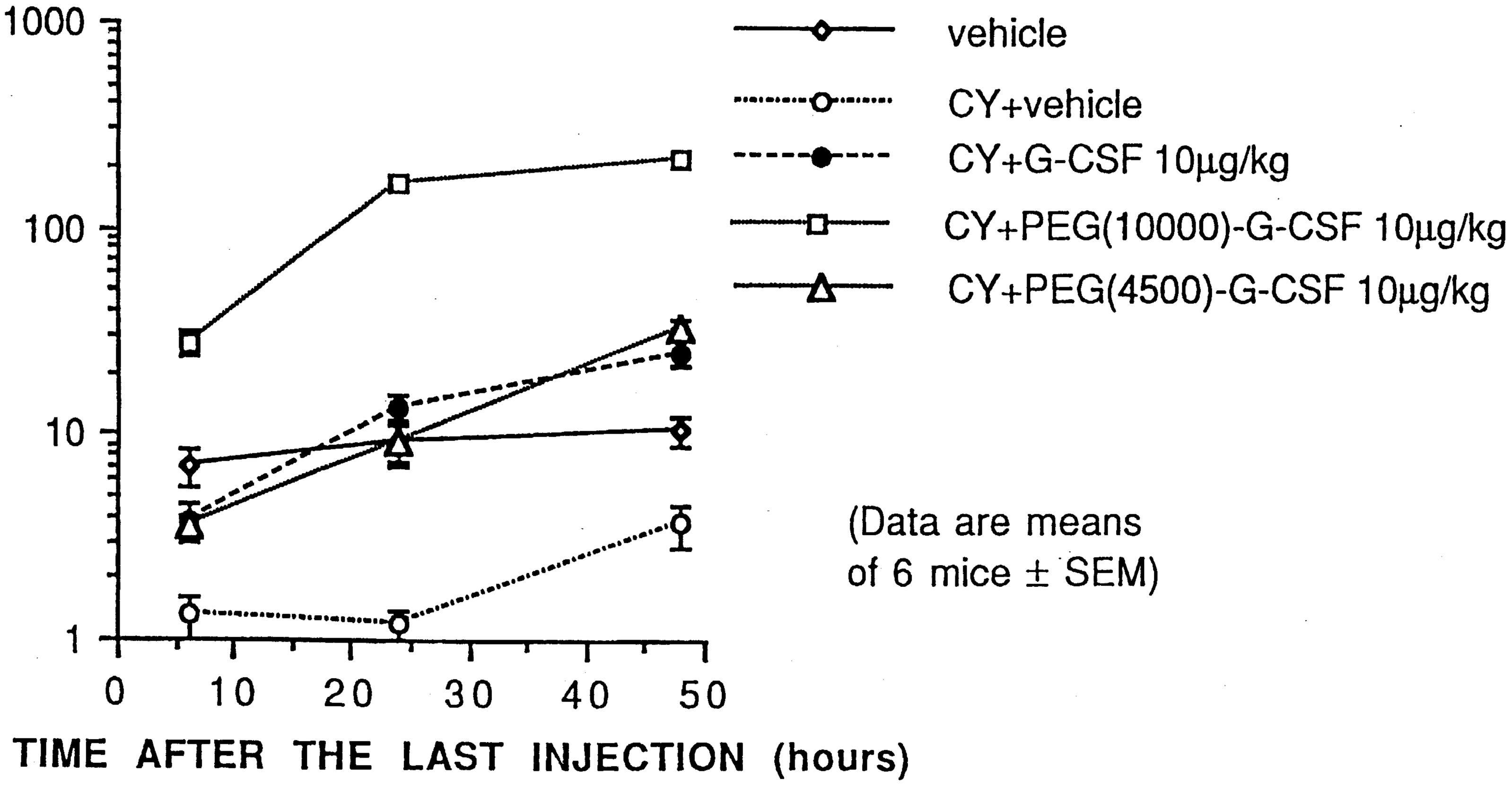


FIG. 4

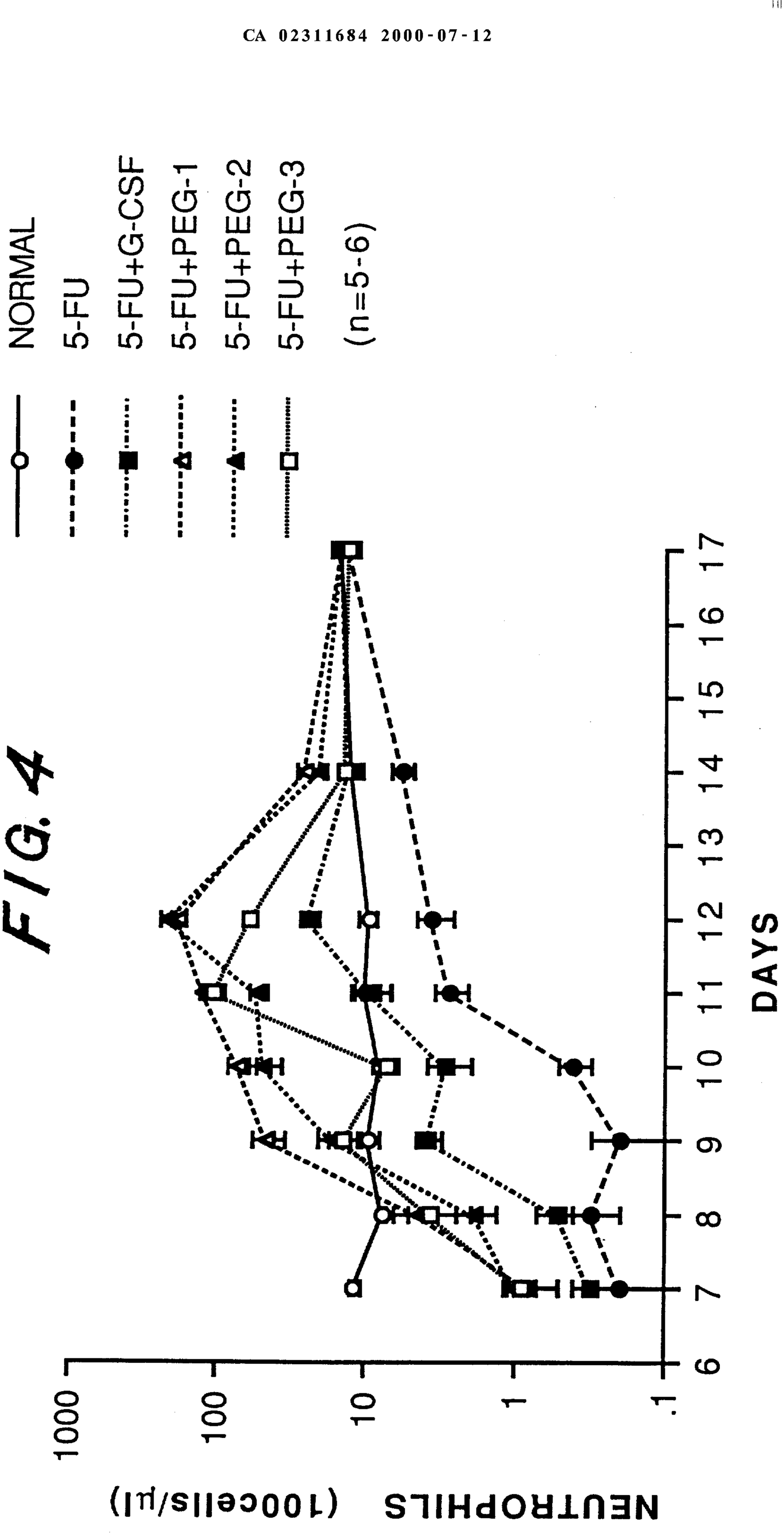
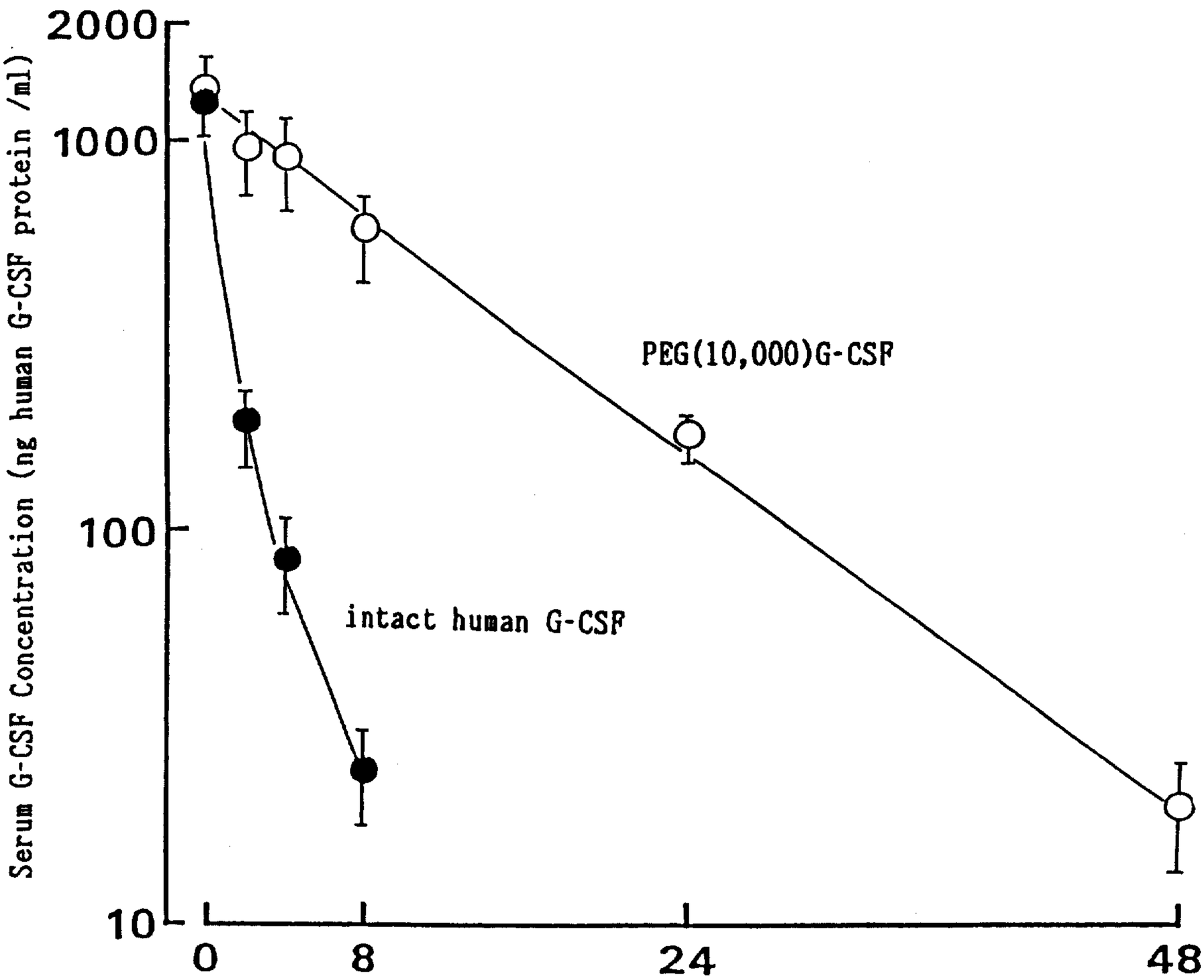
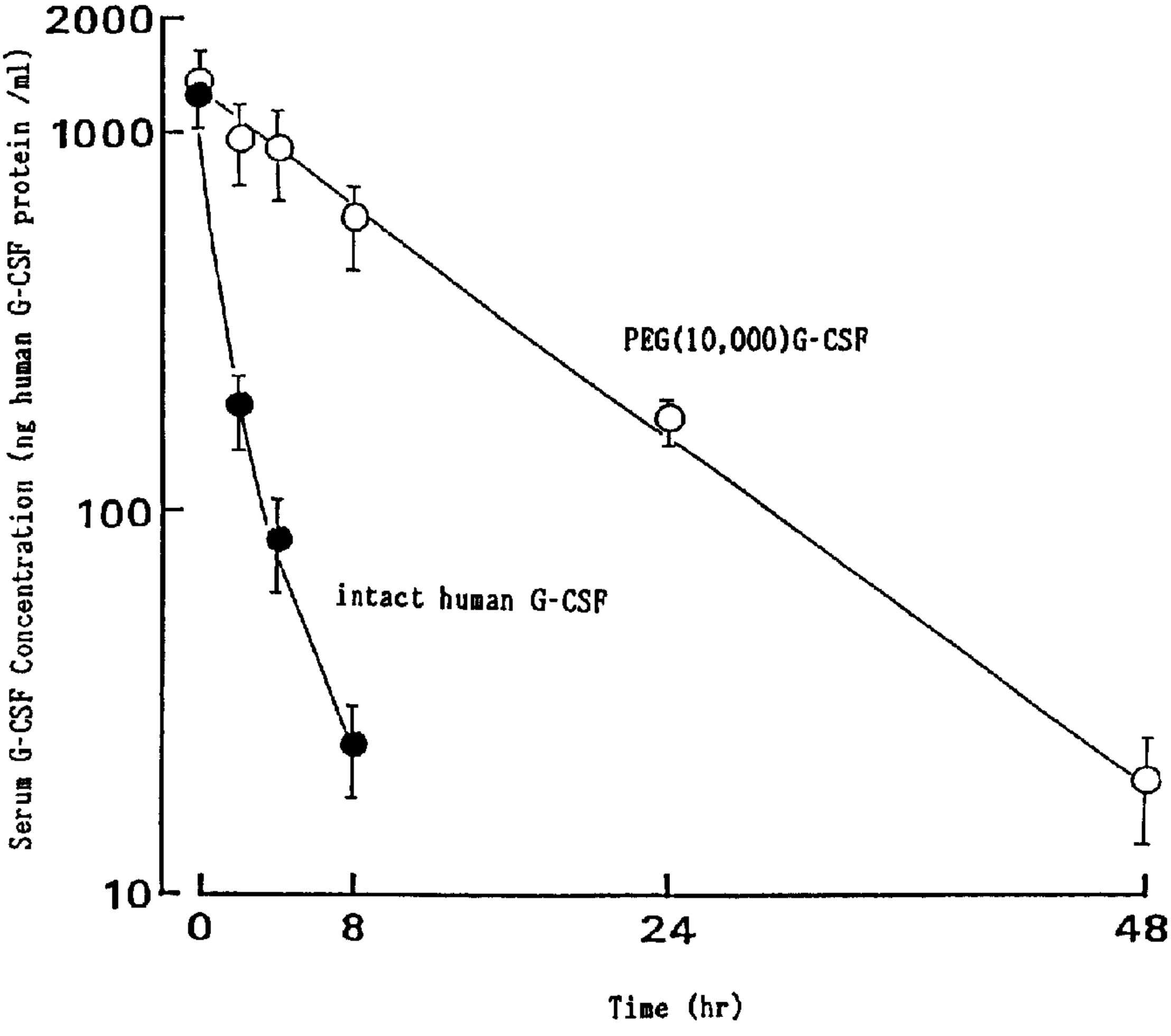

NORMAL
5-FU
5-FU+G-CSF
5-FU+PEG-1
5-FU+PEG-2
5-FU+PEG-3
(n=5-6)
DAYS
7
8
9
10
11
12
13
14
15
16
17
NEUTROPHILS (100cells/μl)
1000
100
10
1
.1

FIG. 5


2000
1000
100
10
Serum G-CSF Concentration (ng human G-CSF protein /ml)
PEG(10,000)G-CSF
intact human G-CSF
0
8
24
48
Time (hr)


2000
1000
100
10
Serum G-CSF Concentration (ng human G-CSF protein /ml)
PEG(10,000)G-CSF
intact human G-CSF
0
8
24
48
Time (hr)