Prolonged Circulation of Recombinant Human Granulocyte–Colony Stimulating Factor by Covalent Linkage to Albumin Through a Heterobifunctional Polyethylene Glycol

Aimee Guider Paige,[1,4] K. Lane Whitcomb,[2] Jennifer Liu,[3] and Olaf Kinstler[1]

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Purpose. Recombinant human granulocyte-colony stimulating factor (rhG-CSF) was covalently conjugated to both rat and human serum albumin (RSA and HSA respectively) to increases the circulating half life ($t_{1/2}$) of rhG-CSF.

Methods. Conjugates of RSA (MW 67,000) and HSA (MW 66,000) were prepared by linking the two proteins through a heterobifunctional maleimido-carboxyl polyethylene glycol (PEG) and were tested in the rat. The conjugates were injected intravenously (IV) at the equivalent dose of 50 μg/kg of rhG-CSF, and white blood cell (WBC) counts and plasma concentrations of drug were determined. A comparison of pharmacokinetic parameters was made between rhG-CSF, the conjugates RSA-PEG-rhG-CSF and HSA-PEG-rhG-CSF, and a non-covalent mixture of rhG-CSF and HSA.

Results. The albumin-rhG-CSF conjugates are eliminated more slowly from the circulation. The clearance values are reduced from 0.839 ± 0.121 ml/min/kg for rhG-CSF to 0.172 ± 0.013 ml/min/kg for RSA-PEG-rhG-CSF and 0.141 ± 0.005 ml/min/kg for HSA-PEG-rhG-CSF. WBC counts increased in both absolute number and duration as compared to rhG-CSF alone. The albumin rhG-CSF conjugates had enhanced serum stability relative to free rhG-CSF. The rate of degradation of the albumin conjugates incubated in rat serum at 37°C decreased five fold.

Conclusions. The results from the study show that specific conjugation of rhG-CSF to albumin decreases plasma clearance *in vivo*, causes increased WBC response, and increases serum stability as compared to free rhG-CSF.

KEY WORDS: albumin; granulocyte-colony stimulating factor; polyethylene glycol; protein conjugate; pharmacokinetics.

INTRODUCTION

The hematopoietic growth factor, recombinant human granulocyte-colony stimulating factor (rhG-CSF) has a half life ($t_{1/2}$) of 1.3-4.2 hours in man (1) which necessitates daily injections for the patient. To extend the circulating $t_{1/2}$ of rhG-CSF and to increase the biological response, both rat serum albumin (RSA) and human serum albumin (HSA) were conjugated to rhG-CSF through a polyethylene glycol (PEG) linker. Successfully extending the $t_{1/2}$ may translate to reduced dosing frequency of rhG-CSF to patients.

Albumin has been coupled to small molecules and proteins to prolong circulation (2,3) and to mask toxicity and immunogenicity (4,5). Albumin-Superoxide Dismutase (SOD) conjugates have an extended $t_{1/2}$ up to six hours as compared to the $t_{1/2}$ of five minutes for free SOD (2). Similar results were seen with albumin-growth hormone (GH) conjugates where the $t_{1/2}$ was extended from five minutes to 2-3 hours (3).

Ligands have been linked to albumin non-specifically by spacers such as gluteraldehyde or a carbodiimide (4) through free amino groups on albumin. The disadvantage of this approach is that albumin has 50 or more lysines (depending on the species) and the chemistry results in a wide range of molecular weight conjugates as well as crosslinked aggregates. An improved approach would be to control the size of the conjugate. This could be achieved by specific attachment of the two proteins through the unpaired cysteine at position 34 ($CySH_{34}$) of albumin and to the α-amino terminus of rhG-CSF resulting in a 1:1 albumin:rhG-CSF conjugate.

Polyethylene glycol (PEG) has desirable properties as well as its ability to be functionalized with different reactive groups at each terminus. PEG-protein conjugates are known to be non-immunogenic, non-antigenic and have increased serum lifetimes (7). While it wasn't expected that the PEG linker would significantly alter the pharmacokinetics due to its small molecular weight, from the known properties of PEG, it was reasonable to assume that the PEG would not have a negative effect on the conjugates. For these reasons, PEG was an ideal spacer between rhG-CSF and albumin.

This specific conjugation approach was taken to produce rhG-CSF drugs which had reduced plasma clearance and increased biological response.

MATERIAL AND METHODS

Chemistry

Ethyl Maleimide Radiolabeled Albumins

Albumin was reduced according to the method of Katchalski *et al.* (8) and the number of free sulfhydryls was determined by Ellman's Reagent (Pierce Chemical). A freshly reduced sample of albumin in 1× Dulbecco's phosphate buffered saline (PBS) (Gibco BRL) (10 mg/ml, 1 ml) was added to an eppendorf tube in which a solution of N-(ethyl-$1^{14}C$) maleimide (0.25 ml, 6.25 μmol, 25 μCi) had been evaporated to dryness with a gentle stream of nitrogen. The reaction mixture was stirred for 48 h at 4°C and was then applied to a pre-equilibrated (1× PBS) PD-10 column (Pharmacia). One milliliter fractions were collected and the radioactive counts were determined using a Beckman scintillation counter (S6001C). Absorbance at 280 nm was measured and a trichloroacetic acid (TCA) precipitation was done as follows: A 10 μl aliquot of the radioactive protein solution was diluted 50 fold with 10 mM NaOAc, 0.02% Tween 20, 0.045 g bovine serum albumin (BSA), pH 4.0. To the diluted sample was added 50 μl of 60% TCA, and the mixture incubated

[1] Department of Pharmaceutics and Drug Delivery, Amgen Inc., Thousand Oaks, California.
[2] Department of Anatomical Pathology, Amgen Inc., Thousand Oaks, California.
[3] Department of Protein Structure, Amgen Inc., Thousand Oaks, California.
[4] To whom correspondence should be addressed at Amgen Inc., 8-1-C, 1840 DeHavilland Drive, Thousand Oaks, California 91320.

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at 4°C for 10 min. After centrifuging the samples at 14,000 rpm for 15 minutes, a sample of the supernatant was removed and analyzed for radioactivity by liquid scintillation.

Albumin-PEG-GLY

Amino-carboxy PEG was prepared from PEG diol (MW 2000, Aldrich) as reported by Zalipski and Barney (9). Under nitrogen, N-γ-malemidobutyryloxysuccinimide ester (Pierce Chemical, 105 mg, 0.375 mmol) was combined with the amino-carboxy PEG (500 mg, 0.25 mmol) and triethylamine (53 μl, 0.375 mmol) in dichloromethane (10 ml). The reaction was stirred for four hours at 22°C and the resulting derivatized PEG was precipitated in cold ethyl ether. The solid was collected, dissolved into water and purified by gel filtration chromatography (Sephadex G-25, Sigma Chemical) eluting with water at 170 cm/h. Fractions were analyzed by a polyacrylic acid test (9) to detect PEG. Fractions containing PEG were combined and lyophilized overnight. The maleimide-carboxy functionalized PEG (345 mg, 0.173 mmol) was dissolved in dichloromethane (6 ml) under nitrogen and was treated with N-hydroxysuccinimide (59 mg, 0.519 mmol) and dicyclohexylcarbodiimide (107 mg, 0.519 mmol) and was stirred at 22°C for 24 h. The precipitate was filtered off, and the filtrate containing bifunctional PEG was precipitated into cold ethyl ether. The PEG activated ester (ester content 32% mol/mol as determined by basic hydrolysis) (2 mg, 0.32 μmol), a solution of (Glycine-1-^{14}C)-ethyl ester hydrochloride (DuPont NEN, 0.15 ml, 0.32 μmol, 15 μCi) in ethanol and triethylamine (0.5 μl, 3.2 μmol) were combined and stirred for 20 h at 22°C. The reaction mixture was placed on a gel filtration column (P-2, Bio-rad) and the radiolabeled PEG was eluted at 170 cm/h with water. Fractions were analyzed for the radioactive counts by liquid scintillation. The product (1 mg, 0.5 μmol) was reacted with freshly reduced serum albumin (human or rat, Sigma Chemical, 0.6 μmol) in PBS for two days at 4°C. Purification was done on Sephadex G-25 (Sigma Chemical) eluting with 1× PBS. Fractions were collected and measured for radioactivity content and absorbance at 300 nm. The purity of the conjugate was determined by TCA precipitation.

Albumin-PEG-G-CSF Conjugates

For a schematic view of the synthesis, see Figure 1. A commercially available 3.4 kDa amino-carboxyl heterobifunctional PEG (Shearwater Polymers) was functionalized as the 2 kDa PEG was to react with the sulfhydryl of albumin and with the α-amino group of G-CSF. The derivatized maleimido-PEG-N-hydroxysuccinimide ester was reacted with E. coli derived rhG-CSF (10). rhG-CSF (80 mg, 4.26 μmol) at 5 mg/ml in 0.10 M $NaPO_4$ at pH 6.0 was added to the activated PEG ester (ester content 52% mol/mol, 120 mg, 18.32 μmol). The reaction was stirred at 4°C for 18 h and diluted to 50 ml with 1 mM HCl. The pH was lowered to 3.5 with 1.0 M HCl. The N-terminally pegylated rhG-CSF was separated from the reaction mixture by ion exchange chromatography (SP Sepharose, 16/10, Pharmacia) eluting with buffer A: 20 mM NaOAc pH 4 and buffer B: A + 1 M NaCl using a linear gradient over 25 column volumes at 30 cm/h. Purified mono N-terminally pegylated rhG-CSF (4.5 mg, 0.241 μmol) was reacted with freshly reduced albumin (RSA and HSA) (ca. 15-20 mg/ml, 0.241 μmol) in 1× PBS at 4°C for 48 hours. The conjugate was purified by preparative size exclusion chromatography (Sudex 200 26/60, Pharmacia) eluting with 50 mM $NaPO_4$ pH 6, 100 mM NaCl at 2.3 cm/h.

Peptide Mapping

Either rhG-CSF (standard) or N-terminally pegylated rhG-CSF was dried in a speed vac, reconstituted in 100 μl of 8M urea, and sonicated for 10 min. After sonication, 10 μl of

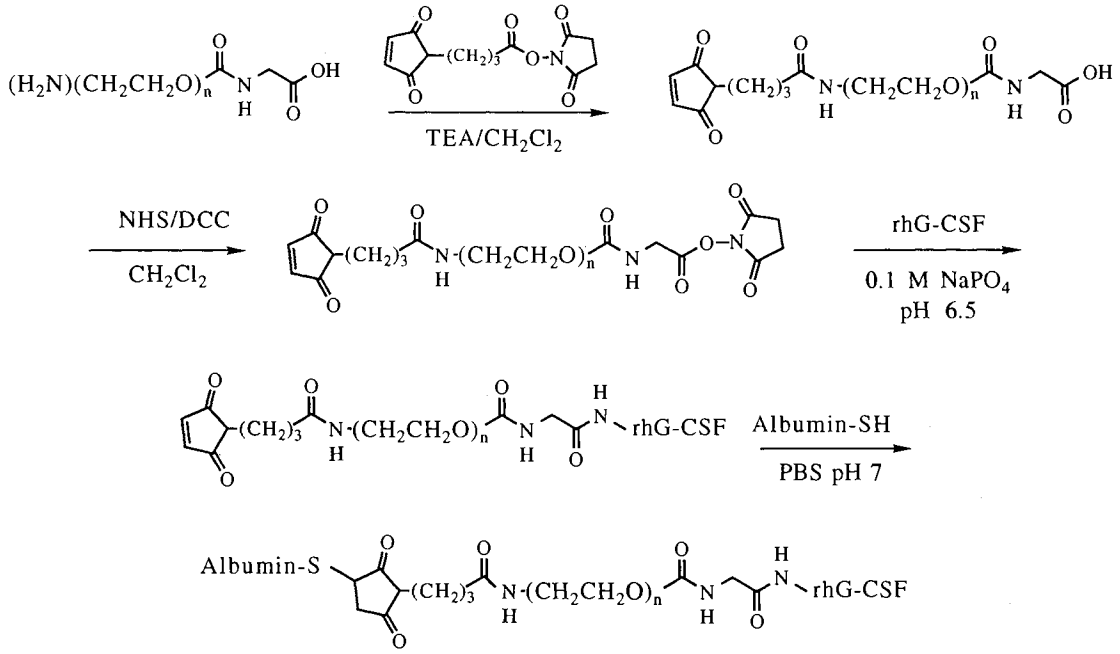

Fig. 1. Synthetic scheme for preparation of rhG-CSF-PEG-albumin conjugates.

1M Tris-HCl, pH 8.5, and 1 μg of Lysyl Endopeptidase (EndoLysC, Wako Chemical) from a 0.1 mg/ml stock solution in 10 mM Tris-HCl, pH 8.5 were added. The total volume was adjusted to 0.2 ml with distilled water, and the proteolytic digestion was carried out for 7 h at 22°C.

Following hydrolysis with EndoLysC, the disulfide bonds were simultaneously reduced with 5 μl of 80 mM tributylphosphine (Aldrich) and alkylated with 10 μl of 40 mM α-(minosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (Wako Chemical) (2 mM final concentration) (10). The reaction mixture was heated at 60°C for 10 min. and then cooled to 22°C.

The generated peptides (0.2 ml) were acidified and injected directly onto a Vydac C_4 (300 Å pore size) column equilibrated with buffer A: 0.1% TFA in water and were eluted with a linear gradient of 3-76% buffer B: 95% CH_3CN, 0.1% TFA in water over 115 minutes. Elution was monitored for absorbance at 215 nm. Individual peptides from rhG-CSF were collected and identified by amino acid compositional analysis and N-terminal sequencing (9).

Animal Experiments

One day prior to dosing, male Sprague Dawley rats (220-270 g, Charles River Breeding Labs, Wilmington, Massachusetts) were anesthetized with an intraperitoneal injection of 90 mg/kg ketamine and 10 mg/kg xylazine. A silastic catheter was inserted into the right jugular vein as described by Niven *et al.* (11). Before dosing, rats were anesthetized with a mixture of 3.5% isoflurane and 2.5 L/min oxygen and then transferred to methoxyflurane nose cones. Baseline blood samples of 250 μl were removed via the indwelling jugular catheter and placed in an EDTA coated eppendorf tube. All drugs were given as a bolus intravenous injection via the penile vein. Doses of 50 μg/kg rhG-CSF in 50 mM $NaPO_4$, 100 mM NaCl, pH 6.0 were given for studies of rhG-CSF, RSA-PEG-rhG-CSF, and HSA-PEG-rhG-CSF. The non-covalent mixture of HSA and rhG-CSF was dosed at 75 μg/kg in the same phosphate buffer. The radiolabeled albumins were injected as a solution in 1× PBS (1 μCi, 1.2-1.5 mg). Blood samples (250 μl) were taken from the catheter at regular intervals. White blood cell counts were determined for samples with an automated cell analyzer (Sysmex F800, Microcell counter, Toa Medical Electronic Co. Kobe, Japan). Samples were kept at 4°C and were spun at 10,000 rpm for 10 minutes. Plasma was decanted and stored at −80°C.

Data Analysis

Pharmacokinetic parameters mean residence time (MRT), plasma clearance (CL_p) and volume of distribution at steady state (V_{ss}) were calculated using standard methods (12).

Plasma concentrations of drug were determined by rhG-CSF enzyme immunoassay (EIA) (Quantikine, R&D Systems). Each drug administered to animals was analyzed separately using a standard curve generated from the drug being detected.

RESULTS AND DISCUSSION

Chemistry

In preparing the conjugates of albumin and rhG-CSF, the main concern was to control the size of the conjugates by controlling the site of PEG attachment. A conjugate of one molecule of albumin to one molecule of rhG-CSF allows for reproducible chemistry, ready characterization, and avoids the large protein aggregates formed with methods previously used for making albumin-protein conjugates. In constructing the conjugate, rhG-CSF is first reacted with activated PEG. By cation exchange chromatography, the different species of pegylated rhG-CSF are separated by the number of PEG molecules attached and by the site of attachment. Furthermore, there is sufficient difference in the pK_a's of the α-amino terminus and the ε-amino group of lysines to target pegylation to the α-amino terminus of proteins in good yield by controlling the reaction pH. α-amino terminus pegylation was confirmed by an EndoLysC peptide map with <5% of rhG-CSF being unmodified at the N-terminus. The purified mono-pegylated rhG-CSF is then reacted with freshly reduced albumin which has only one unpaired cysteine at position 34. Ellmans test verifies one free cysteine per molecule of albumin. The molecular weights of the products were determined to be 81 kDa and 85 kDa respectively for the HSA and RSA conjugates by SDS-PAGE electrophoresis using a Novex 8% Tris/Glycine gel under reducing conditions with Novex Mark12 as molecular weight standards. This is consistent with the theoretical molecular weight for the conjugates of approximately 88 kDa.

Pharmacokinetic Analysis

Initially, reduced albumin (HSA and RSA) was radiolabeled with ethyl maleimide (albumin-EM) to ensure that modification of the $CySH_{34}$ of albumin would not dramatically change the clearance parameters. Figure 2a shows plasma concentration of drug over time and Table I provides pharmacokinetic parameters. While our calculated effective

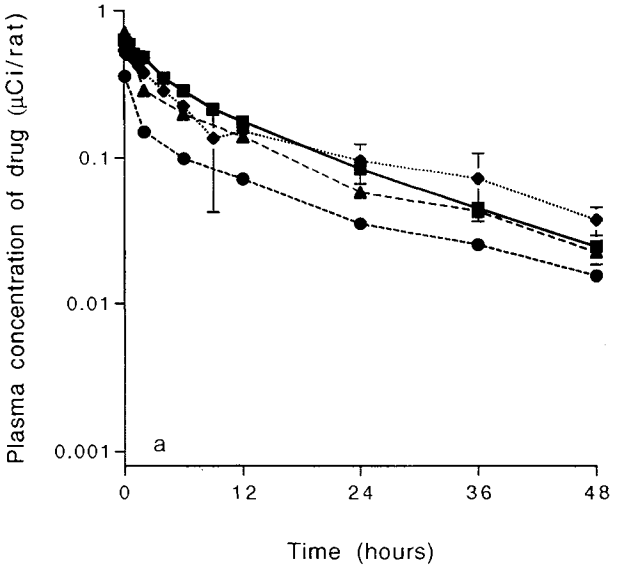

Fig. 2a. Plasma protein concentration of ^{14}C radiolabeled (1 μCi) albumins as determined by percent radioactivity remaining vs time from IV administration of HSA-EM (■) (n = 4), RSA-EM (◆) (n = 4), HSA-PEG-GLY (●) (n = 3), RSA-PEG-GLY (▲) (n = 2). Data shown are ± SD of the mean.

Table I. Pharmacokinetic Parameters in Rats After IV Dosage[a]

Drug	n	Dose (μg/kg)	MRT (min)	Vss (ml/kg)	CLp (ml/min/kg)
RSA-EM	4	477	1393 ± 34	194 ± 11	0.140 ± 0.011
HSA-EM	4	629	1003 ± 107	150 ± 16	0.151 ± 0.024
RSA-PEG-GLY	2	500	957 ± 2	214 ± 7	0.224 ± 0.007
HSA-PEG-GLY	3	480	1033 ± 7	298 ± 5	0.289 ± 0.007
RSA-PEG-GCSF	3	50	394 ± 28	67 ± 2	0.172 ± 0.013
HSA-PEG-GCSF	4	50	425 ± 12	60 ± 3	0.141 ± 0.005
rhG-CSF	8	50	90 ± 16	75 ± 13	0.839 ± 0.121
rhG-CSF + HSA	4	75	124 ± 9	66 ± 2	0.533 ± 0.025

[a] Data shown are the mean ± SD.

$t_{1/2}$ of 16 hours for RSA-EM is significantly below the $t_{1/2}$ of 2.5 days reported in the literature for rats (13), upon examination of the original paper (14), the initial method of $t_{1/2}$ determination ignored the initial alpha phase of drug distribution. In that study, radiolabeled albumin was administered to the animals and two or more days were allowed for equilibrium to be established between all components of the plasma protein pool before any blood samples were taken for pharmacokinetic evaluation.

After determining that the radiolabeled albumins retained prolonged circulating times, radiolabeled pegylated albumins (albumin-PEG-GLY) were constructed and tested in rats (Figure 2a). The plasma clearance values (CL_p) for RSA-EM and HSA-EM are 0.14 ml/min/kg and 0.15 ml/min/kg respectively, and the CL_p values for RSA-PEG-GLY and HSA-PEG-GLY are 0.22 ml/min/kg and 0.29 ml/min/kg respectively. The pegylated albumins are eliminated faster than the radiolabeled albumins, but the CL_p values are still well below the clearance value of 0.84 ml/min/kg determined for rhG-CSF. This indicated that rhG-CSF conjugated to a PEG-albumin could remain in the circulation much longer than native rhG-CSF.

The albumin-PEG-rhG-CSF conjugates were then constructed and tested in rats. Plasma levels of the conjugates RSA-PEG-rhG-CSF and HSA-PEG-rhG-CSF are shown in Figure 2b compared to rhG-CSF. Elimination is slow for the first 12, hours then a more rapid decline follows. Even after 36 hours, the conjugate concentrations are still above that of free rhG-CSF for the same absolute dose of rhG-CSF. The CL_p values for RSA-PEG-rhG-CSF and HSA-PEG-rhG-CSF of 0.17 ml/min/kg and 0.14 ml/min/kg respectively approximate the CL_p values determined for the EM-albumins.

Since albumin is a transport protein that nonspecifically binds many proteins and lipids by hydrophobic interactions, a non-covalent mixture of HSA and rhG-CSF was prepared in a 1:1 molar ratio in the absence of PEG and administered to rats. As is shown in Figure 3, there may be a small positive effect on CL_p due to hydrophobic interactions. The CL_p value of the mixture has decreased to 0.53 ml/min/kg from 0.84 ml/min/kg for unmodified rhG-CSF. However, the dose of rhG-CSF administered for this mixture was 75 μg/kg, and it has been shown by Niven *et al.* that higher doses of rhG-CSF leads to lower plasma clearance (11).

Table I shows a summary of pharmacokinetic data for all drugs administered. In addition to altered CL_p values, the mean residence times (MRT) for the albumin-PEG-rhG-CSF conjugates (6.5 and 7.1 h) are clearly longer than for both free rhG-CSF (1.5 h) and the non-covalent mixture of HSA and rhG-CSF (2.1 h).

Biological Activity

The biological activity was determined by measuring the WBC response in plasma. Figure 4 shows that the peak WBC response for rhG-CSF occurs at approximately 12 hours. The albumin-PEG-rhG-CSF conjugates have peak responses at roughly 36 hours with a 30-60% increase in the peak number of WBCs. Animals treated with the conjugates return to base line measurement 24 hours later than animals treated with unmodified rhG-CSF. The non-covalent mixture of rhG-CSF and HSA shows a slight increase in mean number of cells, but the peak response time has not been extended. The extended response from the conjugates may be expected due to their longer circulation in the blood. It has been shown that injected albumin can be detected in bone marrow megakaryocytes (15) so it is not unexpected that the

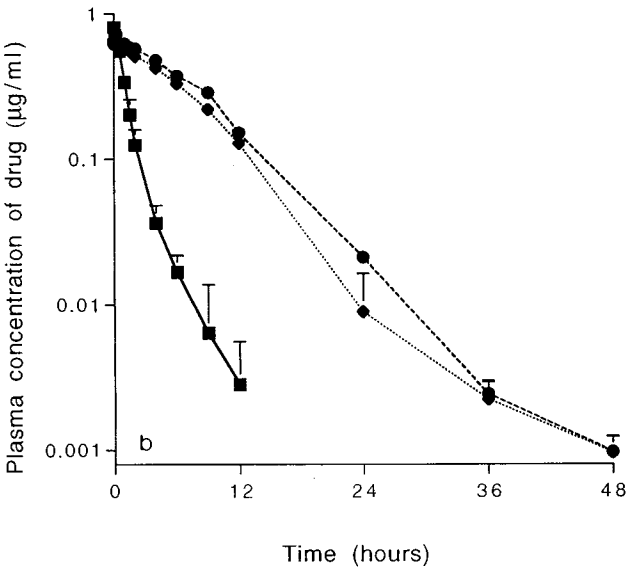

Fig. 2b. Semi-log plot of plasma protein concentration vs time of RSA-PEG-rhG-CSF (♦) (n = 3) and HSA-PEG-rhG-CSF (●) (n = 4) conjugates compared to rhG-CSF (■) (n = 8) after a 50 μg/kg dose IV in rats. Data shown are ± SD of the mean.

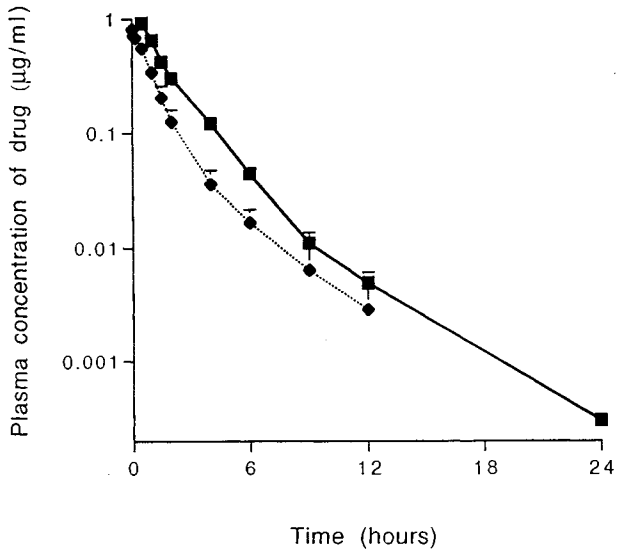

Fig. 3. A semi-log comparison of plasma levels vs time of rhG-CSF (♦) (n = 8) and a non-covalent mixture of rhG-CSF and HSA (■) (n = 4). There is not a large affect on clearance due to a non-covalent association between the two proteins. Data shown are ± SD of the mean.

albumin-rhG-CSF conjugates are able to penetrate the bone marrow and allow rhG-CSF to effect a biological response.

Conjugate Stability

In addition to increasing the circulating $t_{1/2}$ of rhG-CSF, there was the potential that albumin would stabilize rhG-CSF to degradation in serum. This hypothesis was tested by spiking freshly drawn rat serum with either rhG-CSF or RSA-PEG-rhG-CSF and incubating it at 37°C for six days. Fixed volume aliquots were removed at regular intervals, with each sample theoretically containing equal protein concentrations. Analysis of the aliquots by EIA, with standard curves generated for each species, showed an apparent decrease in protein concentration over time for the unconjugated rhG-CSF (Figure 5), indicating degradation. In contrast, the RSA-PEG-rhG-CSF conjugate maintained consistent protein concentration levels even after six days. This suggests that the albumin and/or the PEG had a stabilizing effect on the protein. Another indication that the conjugates remain intact *in vivo* is evident from the EIA data. Since the sensitivity of EIA is reduced for conjugated rhG-CSF relative to free rhG-CSF, an increase in plasma concentration would be seen if free rhG-CSF was being released. Based on these data, it appears likely that an intact conjugate is reaching the bone marrow.

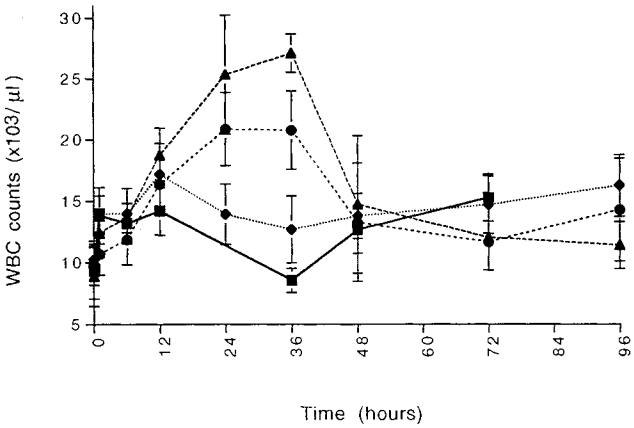

Fig. 4. The WBC response in rats comparing a 50 µg/kg dose of rhG-CSF (■), RSA-PEG-rhG-CSF (●), HSA-PEG-rhG-CSF (▲), and a 75 µg/kg dose of a non-covalent mixture of rhG-CSF and HSA (♦). Data shown are ± SD of the mean (n ≥ 3).

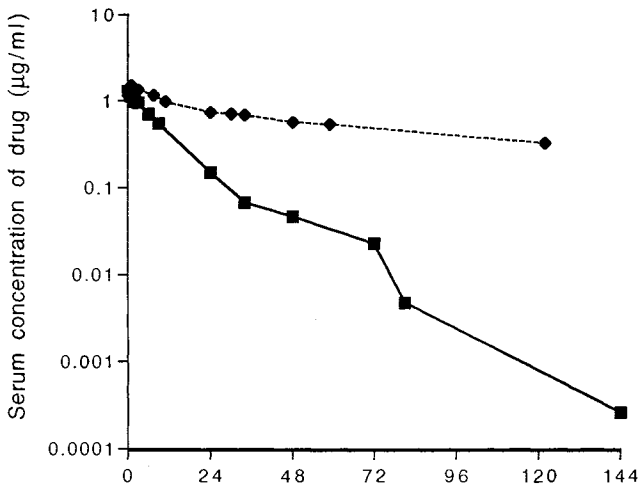

Fig. 5. A semi-log plot comparing apparent protein concentration over time as determined by EIA for rhG-CSF (■) and RSA-PEG rhG-CSF (♦) incubated in rat serum at 37°C.

SUMMARY

Specific conjugation of rhG-CSF to albumin through a heterobifunctional PEG results in a conjugate which remains intact *in vivo* and that increases the circulation of the drug achieving an increased and extended white blood cell response. The longer circulation may be due to the size of the conjugate (95.1 kDa) which is above the cutoff size for glomerular filtration (16) and/or the increased stability of the conjugate in serum. Since the drugs are active, it seems likely that the attachment of the albumin does not interfere with the binding of rhG-CSF and its receptors. In addition, since the chemistry is specific, the drug can readily be characterized and synthesized reproducibly. There is the prospect to use such a conjugate to decrease the frequency of dosing for patients receiving rhG-CSF.

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