Enteral Bioavailability of Human Granulocyte Colony Stimulating Factor Conjugated with Poly(ethylene glycol)

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Purpose. The focus of this paper is to demonstrate that pegylation of a therapeutic protein, recombinant human granulocyte colony stimulating factor (PEG-G-CSF), results in an increase in stability and in retention of *in vivo* bioactivity when administered by the intraduodenal route and may, therefore, be a suitable form of the protein for inclusion in an oral delivery formulation.

Methods. The ability of PEG-G-CSF to elicit a therapeutic response from the enteral route was investigated by two methods of intraduodenal dosing in an *in vivo* model to determine the optimal dosing method: by slow, constant infusion, or by a single bolus administration.

Results. Circulating levels of the proteins confirmed that PEG-G-CSF was delivered into the systemic circulation from the enteral route and that biological activity was retained. Bioavailability from the enteral route by the constant infusion method was calculated from the intravenous administration of the proteins to be between 1.8 and 3.5% while un-modified G-CSF failed to elicit a quantifiable response by this method. Bolus administration of PEG-G-CSF also resulted in biological activity although responses were short lived and significantly lower than with the pegylated formulation.

Conclusions. The possible mechanisms of enteral delivery of PEG-G-CSF are discussed. Our results indicate that oral delivery of pegylated G-CSF may be possible and in fact, preferable to using the un-modified form of the therapeutic.

KEY WORDS: pegylation; recombinant human granulocyte colony stimulating factor; enteral; intraduodenal.

INTRODUCTION

Proteins generally exhibit poor bioavailability (<1%) from the enteral route. Both proteases in the gut and the barrier effect of the intestinal lining (1) have lead researchers to investigate the use of absorption enhancers and derivatized formulations to increase enteral stability. Covalent conjugation of poly(ethylene) glycol (PEG) to proteins (3, 14) has been studied as a method of enhancing oral delivery. Increasing the apparent molecular size of the protein, making it less permeable to the lining of the gut, results in increased resistance to proteases and stabilization to salt and pH (5). Pegylated proteins exhibit an increased circulation time in the body, resulting in greater efficacy compared to non-pegylated proteins (6).

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The objective of this study was to determine the potential of oral delivery of PEG-G-CSF by investigating enteral uptake. An *in vivo* rat model was developed to deliver the proteins directly into the duodenum by either slow, constant infusion or by a single bolus dose. Protein plasma levels and total white blood cell count (WBC's) were measured to determine bioavailability and therapeutic response. An *in vitro* model was established to determine the susceptibility of PEG-G-CSF and G-CSF to trypsin and chymotrypsin proteolysis. The mechanisms by which PEG-G-CSF is able to induce a therapeutic response from the enteral route are discussed.

MATERIALS AND METHODS

Materials

Chemicals and supplies were received as follows: monomethoxypolyethylene glycol, Union Carbide, Charleston, South Carolina; HPLC supplies and Ficoll-Paque gradients, Pharmacia, Piscataway, New Jersey; Biosep SEC-2000 column, Phenomenex, Torrance, California; chymotrypsin and protease inhibitors, Boehringer Mannheim, Indianapolis, Indiana; trypsin, Sigma, St Louis, Missouri; recombinant human G-CSF, Amgen, Inc.

Synthesis of PEG-G-CSF

Synthesis of the N-hydroxysuccinimidyl ester of carboxymethyl methoxypolyethylene glycol (SCM-MPEG, molecular weight 6,000) was based closely upon a previous method (13). A 15-fold molar excess of the SCM-MPEG was added to 100 ml of 10 mg/ml G-CSF in 100 mM Bicine pH 8.0 incubated for 1 hour at room temperature, diluted with deionized water to 500 ml, and pH adjusted to 4.0.

PEG-G-CSF was purified by FPLC with a Toyopearl SP550C (TosoHaas, Montgomeryville, Pennsylvania) column, prewashed with 0.2 N NaOH, pre-equilibrated with column buffer (20 mM sodium acetate buffer pH 4.0) and eluted under step gradient conditions with column buffer containing 1 M NaCl with final elution of 350 ml NaCl. Fractions containing PEG-G-CSF were pooled, concentrated, and buffer exchanged into formulation buffer (10 mM sodium acetate pH 4.0, 5% mannitol, 0.004% Tween 80).

Iodination of PEG-G-CSF

PEG-G-CSF was radiolabeled by a method using Enzymobeads (Pierce, Rockford, Illinois) and incubated at 4°C for 2 hours with 50 μg (50 μl) PEG-G-CSF, 20 mM (250 μl) sodium phosphate buffer pH 6.0, 2% (50 μl) β-D- glucose, 2.5 mCi (25 μl) of $Na^{125}I$ and of 41 μM Nal (25 μl). Reaction was terminated by the addition of 20 μl of 1 mM p-hydroxybenzoate and incubated for 10 minutes at 4°C. The sample was brought to 650 μl with column buffer (10 mM sodium acetate buffer pH 4.0, with 0.02% Tween 80), separated on a pre-equilibrated Sephadex G25 column and dialyzed at 4°C in 10 mM sodium acetate buffer, 0.004% Tween 80, pH 4.0. Protein purity was determined by ammonium sulfate precipitation and labeled PEG-G-CSF was analyzed by size exclusion chromatography (SEC) on a Biosep SEC-2000 column

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(Phenomenex, Torrance, California) with 100 mM sodium phosphate buffer, pH 6.9 for determination of specific activity.

Determination of Bioactivity

In vitro bioactivity of PEG-G-CSF was determined by measuring the stimulated uptake of ^{3}H-thymidine (New England Nuclear, Boston, Massachusetts) into mouse bone marrow cells.

Bone marrow cells from the hind legs of female Balb C mice were purified on a Ficoll-Paque density gradient and cultured in McCoy's 5A medium with 10% FBS, 10 mM sodium pyruvate, 1x minimum essential medium (MEM) amino acids, 40 μM essential amino acids, 0.04% sodium bicarbonate, 1x MEM vitamin solution, 10 mM L-glutamine and 0.005% gentamicin sulfate. After incubation for 2 hours at 37°C under 5% CO_2, non-adherent cells were collected in the supernatant and the number of viable cells were counted.

Standard or test material with culture medium containing 4×10^4 non-adherent mouse bone marrow cells were added to a 96 well plate. Following incubation for 68 hours at 37°C under 5% CO_2, 0.5 μCi of thymidine [methyl-^{3}H] was added to each well. Plates were incubated an additional 5 hours. Cells were collected on filter paper, rinsed with water (10x) and EtOH (1x) and counted in scintillation fluid in a beta plate scintillation counter (LKB model 1205-001).

In vivo bioactivity was measured in Male Golden Syrian Hamsters injected with G-CSF or PEG-G-CSF subcutaneously while vehicle control group received 1 mM HCl. Hamsters were sacrificed by CO_2 inhalation at 12, 24, 48 and 72 hours following injection. Blood samples were obtained by cardiac puncture. WBC's were determined using a Sysmex F-800 microcell counter (Baxter, Irvine, California).

In Vitro Proteolysis

G-CSF and PEG-G-CSF (100 μg/ml) were incubated with trypsin or chymotrypsin (1 μg/ml) at 37°C. At 0, 0.25, 0.5, 1, 2 and 4 hours, sample was withdrawn (200 μl) and added to 4°C protease inhibitor cocktail (9 μl), consisting of 20 μg of N-tosyl-L-lysine chloromethyl ketone (TLCK), 16 μg of (4-amidinophenyl) methanesulfonyl fluoride (APMSF), and 1 IU of α_2-macroglobulin.

G-CSF and PEG-G-CSF were analyzed under reducing conditions as previously described (15) by SDS-PAGE (Integrated Separation systems, Natick, Massachusetts). After transferring the proteins onto Immobilon®, protein was detected by incubation with rabbit polyclonal antibody to rhG-CSF followed by incubation with ^{125}I labeled protein A (Amersham, Arlington Heights, Illinois) as a secondary probe for detection of IgG. Quantitation of the remaining protein was achieved by using an autoradiograph as a template to cut and count the Immobilon®.

Enteral Administration

Male Sprague-Dawley rats weighing 250-350 grams (Simonsen), were jugular-cannulated for later blood sampling. Patency of cannulae was maintained by periodic flushing with heparinized saline (30 U/ml). Following two days of recovery from surgery, the stomach was exposed (under 50 mg/kg, Sodium Pentobarbital anesthesia, i.p.) through a midline incision just below the xiphoid process. A purse string suture was made in the duodenum, 1 cm distal to the pylorus. A 2 mm incision was made in the center of the suture to allow the advancement of a 10 cm silastic catheter.

For 24 hour infusion of protein, the cannula was secured in place by closing the purse string suture taking care to assure that the cannula was not occluded by this procedure. An Alzet® (Alza, Palo Alto, California) mini pump, model 2001D delivering 9 μl/hour/24 hours, was prefilled under sterile conditions with the indicated dose of protein. The pump was secured to the free end of the cannula and placed in the peritoneal cavity. The length (8cm) of the cannula into the duodenum and the return of peristalsis following recovery of the rats from anesthesia, rendered leakage of the protein into the peritoneum an unlikely event. Abdominal musculature was closed with a running 4-0 silk suture and skin closed with wound clips. Over the 24 hour duration of pump operation, a total enteral dose of 823 μg/kg of PEG-G-CSF or 755 μg/kg of G-CSF was administered. In the same study, i.v. administration of the proteins was conducted.

Rats also were administered PEG-G-CSF and G-CSF by a single i.d. bolus rather than the slow, constant infusion method of the osmotic pump. The bolus experiment was performed exactly as the infusion studies with the exception of the method of administration. For i.d. bolus administration, the proteins were injected into the duodenum through the free end of the cannula which was then withdrawn following administration of the protein and the purse string suture was closed.

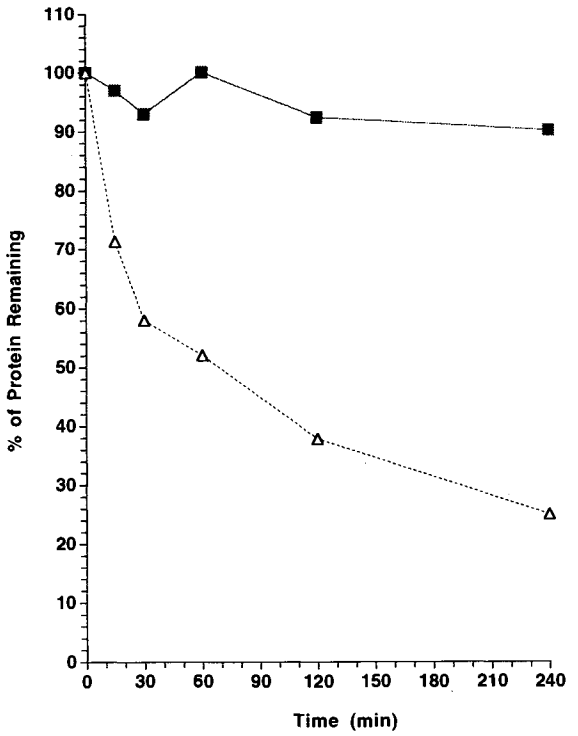

Fig. 1. Effect of pegylation on the relative susceptibility of PEG-G-CSF (■) and G-CSF (△) to trypsin proteolysis in the *in vitro* assay is shown. The data points are each derived from one observation and all the time points are from the same incubation.

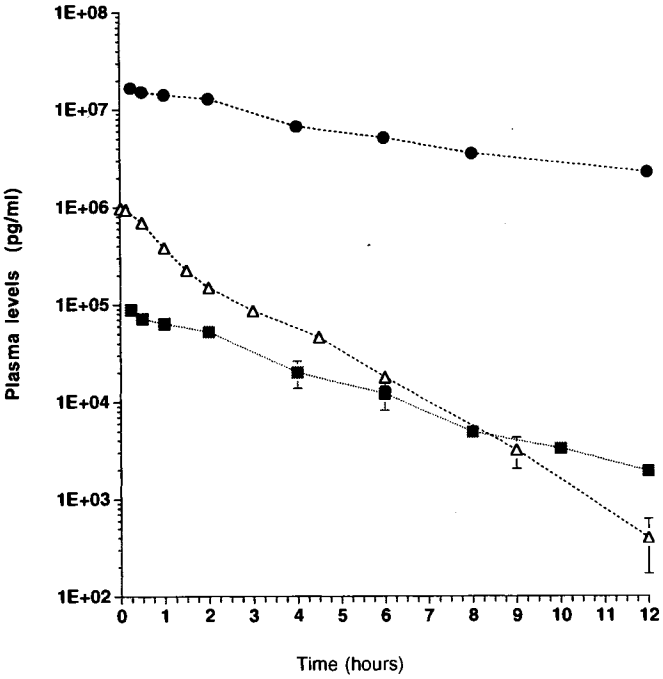

Fig. 2. Effect of pegylation on the plasma clearance of G-CSF in the rat. Plasma concentration of G-CSF was determined following i.v. administration of PEG-G-CSF at 5.96 μg/kg (■) and at 594 μg/kg. (●) and for G-CSF at 50 μg/kg (△). The data points are the mean ± SEM (n = 4).

For the intravenous infusion of proteins, jugular cannulated rats were administered the protein in formulation buffer through the penile vein.

Sampling and Analysis

Blood samples collected in EDTA were centrifuged at 12,000 rpm for 15 minutes. Separated plasma was stored at −80°C pending analysis by ELISA (R & D Systems, Minneapolis, Minnesota).

Pharmacokinetic analysis was performed using the MINSQ II program (Micromath, Salt Lake City, Utah). The area under the plasma concentration-time curve (AUC) and the WBC-time curve were calculated according to the linear trapezoidal rule. Area under the first moment curve (AUMC) was determined in an analogous manner. Plasma clearance (Cl_p) of the proteins was calculated as Dose/AUC. An elimination half-life ($t_{1/2}$) for the PEG-G-CSF was calculated as $t_{1/2} = 0.693 \times MRT$ where MRT is the mean residence time of the protein in the plasma and is derived from the area under the first moment curve (AUMC) divided by the AUC. Levels of statistical significance were assessed using the unpaired, 2-tailed Student's t-test and significance assumed as $p < 0.05$.

RESULTS

Characteristics of PEG-G-CSF

The G-CSF molecule contains a total of five possible sites for covalent attachment of PEG molecules: four lysine residues and the N-terminus. The apparent molecular size and the degree of pegylation were determined using SDS-PAGE with Coomassie Blue staining. The percent of mono, di, tri and tetra pegylation was determined to be 3.4, 31.9, 49.3 and 15.4 percent, respectively. The high degree of pegylation of G-CSF resulted in a decrease in *in vitro* bioactivity, 9%, as compared to the un-modified species. This apparent loss of receptor binding was more than compensated for *in vivo* by the increased circulation time of PEG-G-CSF resulting in *in vivo* bioactivity of 265% ± 10%.

Proteolysis

Quantitation of percent remaining G-CSF following incubation with trypsin, by SDS-PAGE and autoradiography, showed an apparent susceptibility to trypsin proteolysis (Figure 1). After 4 hours of incubation, >90% of PEG-G-CSF was remaining whereas, within 1 hour, ≈50% of G-CSF appeared degraded under these same conditions. When PEG-G-CSF and G-CSF were incubated with chymotrypsin under the same conditions, degradation was not measurable (data not shown).

Pharmacokinetic Analysis

Following i.v. administration of either protein, a mono-exponential curve fit on the PEG-G-CSF data and a bi-exponential fit on the G-CSF data determined that clearance of PEG-G-CSF from the plasma (Cl_p) decreased with increased dose. Differences in clearance were reflected in the

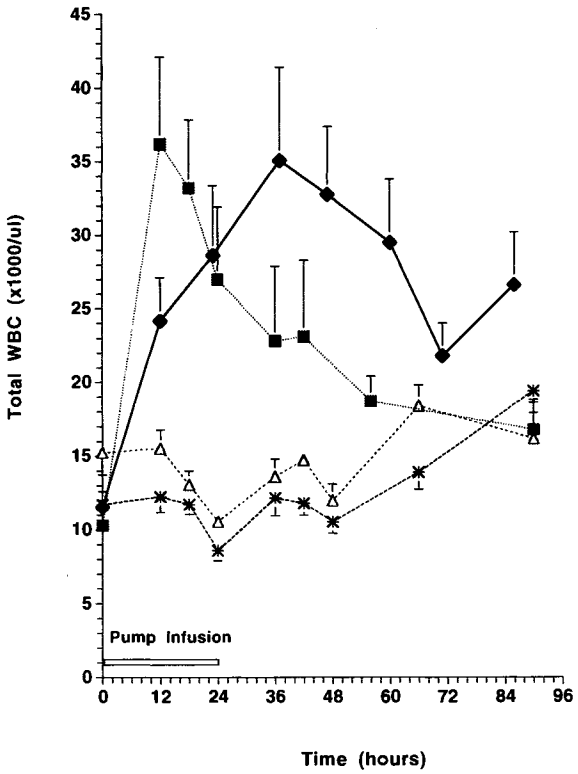

Fig. 3. Enteral infusion of PEG-G-CSF and G-CSF in the rat. Total WBC response in rats i.v. infused with PEG-G-CSF at 50 μg/kg (♦), i.d. infused at 823 μg/kg (■), G-CSF i.d. infused at 755 μg/kg (△) and i.d. infusion of vehicle control group (1 mM HCl) (✱). Data points are the mean ± SEM (n = 4).

Table I. Bioequivalence and Bioavailability of PEG-G-CSF and G-CSF From the Enteral Route

Protein	Dosing	Dose μg/kg	Bioavailability[a] Average AUC_{hrs} (pg · hr/ml)	Bioavailability[a] %	Bioequivalence[b] Average AUC_{hrs} (WBC's · hr/ml)	Bioequivalence[b] %
G-CSF	i.v. Infusion	25	$AUC_{90} = 2 \times 10^5$	100	AUC_{90} = 1488	100
G-CSF	i.d. Infusion	755	AUC_{90} = ND	0	AUC_{90} = None	0
PEG-G-CSF	i.v. Infusion	50	$AUC_{90} = 2.2 \times 10^6$	100	AUC_{90} = 1136	100
PEG-G-CSF	i.d. Infusion	823	$AUC_{90} = 6.3 \times 10^5$	1.8 ± 1.1	AUC_{90} = 852	4.6 ± 1.3
G-CSF	i.v. Bolus	50	$AUC_{24} = 7.2 \times 10^7$	100	AUC_{24} = 216	100
G-CSF	i.d. Bolus	500	$AUC_{24} = 1.8 \times 10^3$	2.5×10^{-4}	AUC_{24} = 40	1.9 ± 0.35
PEG-G-CSF	i.v. Bolus	5.96	$AUC_{24} = 2.7 \times 10^5$	100	AUC_{24} = 234	100
PEG-G-CSF	i.d. Bolus	500	$AUC_{24} = 4.5 \times 10^4$	0.17 ± 0.1	AUC_{24} = 156	0.8 ± 0.08
^{125}I-PEG-G-CSF	i.v. Infusion	0.66	$AUC_{48} = 7.1 \pm 0.9 \times 10^4$	3.5 ± 0.24		
	i.d. Infusion	0.73	$AUC_{48} = 2.7 \pm 0.2 \times 10^3$			

[a] Bioavailability by the enteral route as determined from the $AUC_{(hrs)}$ from plasma concentrations.
[b] Bioequivalence as a measure of the therapeutic response determined from the $AUC_{(hrs)}$ for the total WBC response. ND = Not Detectable by the R & D Systems ELISA method. Results are the mean values from where n = 4, ±SEM where shown.
Area under the plasma level-time curve over 48 hours (AUC_{48}) for TCA-precipitable ^{125}I-labelled PEG-G-CSF in plasma determined for each rat (n = 4).

$t_{1/2}$ of the proteins and were determined to be 1.94 hours and 2.3 hours for PEG-G-CSF when dosed at 5.96 μg/kg and 594 μg/kg respectively compared with 0.95 hours for G-CSF.

Enteral Delivery by Slow Infusion

Formulations of PEG-G-CSF and un-modified G-CSF were prepared for use in Alzet® mini osmotic pumps. Following i.d. infusion of PEG-G-CSF, high plasma levels of the protein were attained, however, i.d. administration of PEG-G-CSF did not appear to reach steady state as the i.v. infused protein did. Un-modified G-CSF, infused in the same manner as PEG-G-CSF, failed to produce detectable concentrations of circulating G-CSF.

Plasma levels of the proteins achieved are reflected in the therapeutic responses seen in Figure 3. Greater than

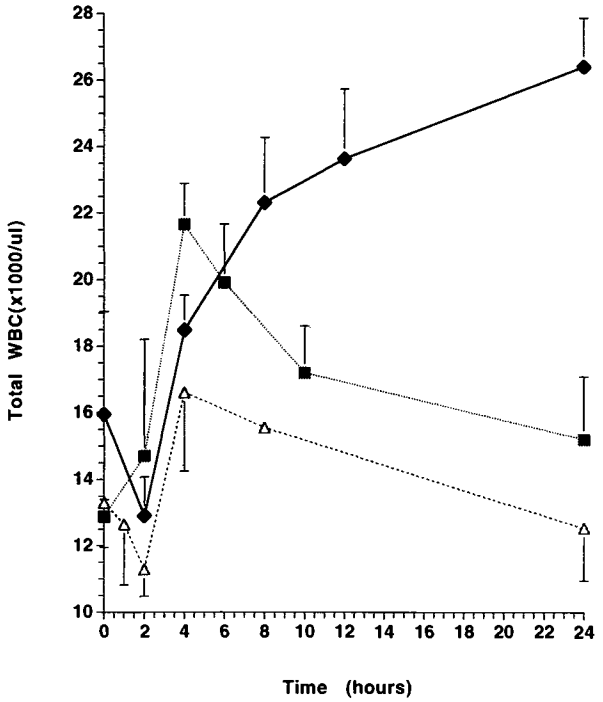

Fig. 4. WBC response to G-CSF following i.v. bolus administration of PEG-G-CSF at 5.96 μg/kg (◆), i.d. bolus administration at 500 μg/kg (■), and G-CSF by i.d. bolus administration at 500 μg/kg (△). Data points are the mean ± SEM (n=4).

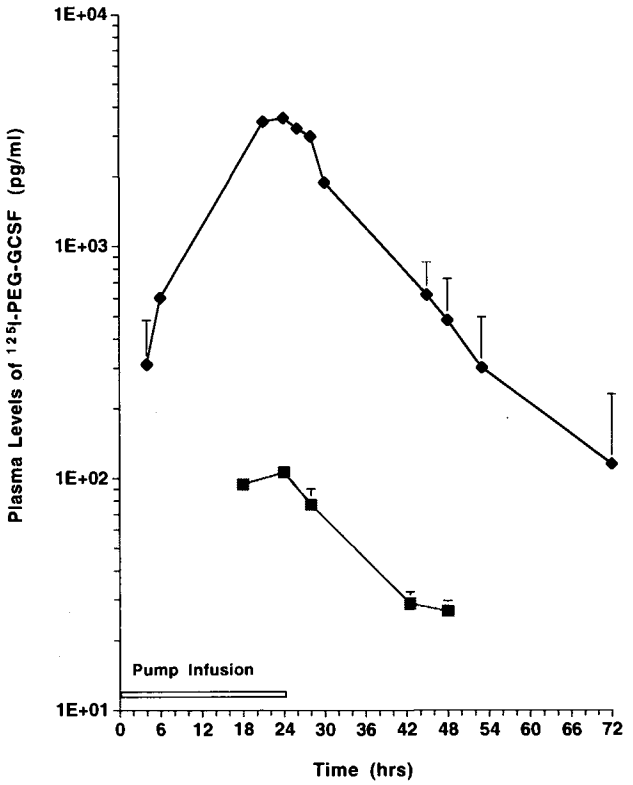

Fig. 5. Enteral infusion of ^{125}I-labeled PEG-G-CSF. Plasma levels of TCA-precipitable ^{125}I-labeled PEG-G-CSF are shown following i.v. infusion of PEG-G-CSF at 0.66 μg/kg (◆) and by i.d. infusion at 0.73 μg/kg (■). For the i.d. infused material, plasma levels became undetectable after 48 hours. Results are the mean ± SEM (n=4).

three-fold elevation of WBC's were measured following i.v. infusion of PEG-G-CSF. A peak WBC response was observed at 36 hours following initial dosing compared to a WBC peak following i.d. infusion, at 12 hours. Rats receiving G-CSF failed to elicit a detectable elevation of WBC's, not unexpectedly, given the undetectable concentrations of protein in the plasma. Rats receiving the vehicle control (1 mM HCl) failed to elicit measurable elevations in WBC's indicating that neither the surgery nor the exposure to the pump itself, had an immediate affect on the WBC levels.

Bioavailability and bioequivalence of i.d. infused G-CSF (Table I) was not detectable, however, results calculated for PEG-G-CSF demonstrated bioavailability and bioequivalence of 1.8% and 4.6% respectively.

Enteral Delivery by Bolus Administration

Plasma levels were measurable for both PEG-G-CSF and G-CSF. PEG-G-CSF dosed animals maintained measurable plasma levels for 24 hours. Circulating protein concentrations were variable and low with G-CSF, with plasma levels undetectable just 6 hours after dosing.

The WBC response to i.d. bolus PEG-G-CSF increased 1.7 fold over initial counts (Figure 4). Response to unmodified G-CSF administered by i.d. bolus was negligible. The vehicle control group (data not shown) failed to exhibit a measurable increase in WBC's. Bioavailability, calculated from i.v. bolus administration of PEG-G-CSF, and bioequivalence for i.d. bolus administration, were determined from the data up to 24 hours. Table I illustrates the comparison between the pump infusion and the i.d. bolus method. Response to bolus administered PEG-G-CSF was not nearly as pronounced as with the pump infusion method of administration.

Enteral Infusion of ^{125}I-PEG-G-CSF

To determine why plasma levels did not reach steady state after i.d. infusion of PEG-G-CSF, a low, non-therapeutic, dose of the radiolabeled protein was evaluated. TCA precipitated protein was quantified and considered as indicative of ^{125}I-PEG-G-CSF plasma concentration. Results indicate that the i.v. and i.d. infused protein do achieve both peak plasma concentrations and plasma steady state after $\approx$18 hours of infusion and plasma concentrations do indeed, remain at steady state for 24 hours. Bioavailability of ^{125}I-PEG-G-CSF from the enteral route is summarized in Table I.

DISCUSSION

Four our purposes, enteral administration, using the Alzet® mini osmotic pump as a model for infusing a relatively constant amount of the drug into the duodenum, would be reflective of a sustained release formulation. Many enteric coatings are available for delivering therapeutics to the intestine (8) and could be incorporated into an eventual formulation for optimal uptake.

The manner in which the PEG-G-CSF molecule may be able to elicit a therapeutic response from the enteral route can be summarized into three possible scenarios.

The first is that protection against proteolysis allows the protein to remain in an active form in the gut before it is absorbed. While gut proteases, specifically trypsin and chymotrypsin, serve as major obstacles to oral delivery of a therapeutic protein, increased protease resistance of pegylated proteins has been previously reported (4-5). While it would be difficult to achieve conditions *in vitro* that exactly represent the *in vivo* state due to the variable dilution effect and competition by other proteins for the trypsin active site in the gut, it is probably safe to assume that the conditions in the assay are more likely to cause degradation than would be observed *in vivo*. Our results suggest that pegylation of G-CSF confers resistance to trypsin degradation whereas un-modified G-CSF indicates an apparent susceptibility to trypsin proteolysis under the same conditions. The active, pegylated form of G-CSF would therefore, seem more likely to survive trypsin proteolysis in the gut.

An additional determinant contributing to circulating therapeutic levels of PEG-G-CSF from the enteral route, is decreased clearance of the protein. Glomerular filtration and metabolism in the proximal tubes seems to be the major route of elimination of G-CSF (13) but it is well known that the larger the protein the slower the renal clearance allowing the protein to circulate longer in the systemic circulation. The high degree of PEGylation of the tested PEG-G-CSF suggests that glomerular filtration will be minimal and clearance is likely to be effected via other mechanisms.

Thirdly, pegylation of the protein may alter the permeability of the mucous and or epithelial layer. Intestinal permeability to PEG molecules has been investigated (30-32) suggesting that uptake is due to solvent drag through intracellular channels in the epithelia of the small intestine.

To reduce to practice the oral administration of PEG-G-CSF, a formulation of the drug would require slow, constant release of PEG-G-CSF, incorporation of an enhancing factor, and an enteric coating designed to release the drug to the optimal site of uptake in the gut.

Our work with PEG-G-CSF has demonstrated that the pegylated formulation is able to not only enter the systemic circulation from the enteral route but that it does so in an active form when delivered by slow, constant infusion. The un-modified protein failed to produce a significant WBC response from the enteral route. The data presented here suggests some potential mechanisms that could account for the results observed and give encouragement for the eventual oral delivery of recombinant therapeutic proteins.

ACKNOWLEDGMENTS

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