The Pulmonary Absorption of Aerosolized and Intratracheally Instilled rhG-CSF and monoPEGylated rhG-CSF

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Purpose. The objective of this study was to highlight differences in the pulmonary absorption of a monoPEGylated rhG-CSF and rhG-CSF after intratracheal instillation and aerosol delivery. ***Methods.*** Male Sprague Dawley rats (250 g) were anesthetized and intratracheally instilled (IT) with protein solution or were endotracheally intubated and administered aerosol for 20 min via a Harvard small animal ventilator. A DeVilbiss "Aerosonic" nebulizer containing 5 ml of protein solution at ≈ 3 mg/ml was used to generate aerosol. The volume of protein solution deposited in the lung lobes was estimated to be ≈13 μl after delivery of Tc-99m HSA solutions. The PEGylated proteins consisted of a 6 kDa (P6) or 12 kDa PEG (P12) linked to the N-terminus of rhG-CSF. rhG-CSF also was administered IT in buffers at pH 4 and pH 7 and in dosing volumes ranging from 100 to 400 μl. Blood samples were removed at intervals after dosing and the total white blood cell counts (WBC) were determined. Plasma was assayed for proteins by an enzyme immuno assay. ***Results.*** The plasma protein concentration *v.* time profiles were strikingly different for aerosol *v.* IT delivery. The C_{max} values for rhG-CSF and P12 after aerosol delivery were greater than found after IT (Aerosol: 598 ± 135 (ng/ml) rhG-CSF; 182 ± 14 P12 *v.* IT: 105 ± 12 rhG-CSF; 65.9 ± 5 P12). Similarly, T_{max} was reached much earlier after aerosol administration (Aerosol: 21.7 ± 4.8 (min) rhG-CSF; 168 ± 31 P12 *v.* IT: 100 ± 17 rhG-CSF; 310 ± 121 P12). Estimated bioavailabilities (F_{lung} %) were significantly greater via aerosol delivery than those obtained after IT (Aerosol: 66 ± 14 rhG-CSF; 12.3 ± 1.9 P12 *v.* IT: 11.9 ± 1.5 rhG-CSF; 1.6 ± 0.1 P12). An increase in circulating WBC counts was induced by all proteins delivered to the lungs. The rate and extent of absorption of rhG-CSF was not influenced by the pH employed nor the instilled volume. ***Conclusions.*** Estimates of bioavailability are dependent upon the technique employed to administer drug to the lungs. Aerosol administration provides a better estimate of the systemic absorption of macromolecules.

KEY WORDS: aerosols; G-CSF; lung; drug delivery; regulation; proteins.

INTRODUCTION

Studies have shown that recombinant human granulocyte colony stimulating factor (rhG-CSF) (1,2) and heterogeneously polyethylene glycolated rhG-CSF (PEG rhG-CSF) (3) introduced to the lungs via intratracheal instillation (IT) are absorbed and provoke a systemic response. Unfortunately, information derived about the rate and extent of absorption is heavily dependent on the method of delivery. Instillate is deposited predominantly in the central, conducting regions of the lung whereas aerosol administration results in more homogeneous deposition of drug throughout the lungs (4,5).

Dosing of proteins by aerosol is also problematic. Difficulties include: a) determining the dose that deposits in the lungs, b) administering a useful dose in a short period of time, c) ensuring stability of the protein during aerosolization and, d) obtaining sufficient protein to conduct the studies. Dosing of aerosol to rodents in pre-clinical studies requires the use of a continuous aerosol dosing system: typically a nebulizer or 'dust' feeder. However, most exposure systems are expensive, operator intensive, and are primarily designed for toxicology.

One approach that avoids some of the above problems involves aerosol administration to anesthetised and ventilated animals (6). The additional use of an in-line ultrasonic nebulizer (7) allows delivery of high aerosol mass concentrations in conjunction with controlled breathing cycles. In this study a similar method has been employed to study the aerosol pharmacokinetics of rhG-CSF and monoPEGylated rhG-CSF in rats. A comparison is made between the pharmacokinetic and pharmacodynamic data obtained with the PEGylated compounds and the unconjugated protein. Similarly, the aerosol results are compared to IT data obtained after varying the buffer composition and volume of instilled solutions.

MATERIALS AND METHODS

Preparation and Characterization of monoPEGylated rhG-CSF

The rhG-CSF was obtained from Amgen manufacturing (lot T6803) at a concentration of 4 mg/ml in 1 mM HCl pH 4. The protein was chemically modified with an activated methoxyPEG-derivative at the primary α-amino groups of the N-terminal residue or rhG-CSF. A detailed discussion of the preparation and characterization of the monoPEGylated conjugates will be described elsewhere. Briefly, rhG-CSF was reductively alkylated with methoxy-PEG-aldehyde (MW 6 kDa or 12 kDa) in the presence of a reducing agent. The mono-methoxyPEG-rhGCSF derivative (PEG rhG-CSF) was isolated from the reaction mixture by ion exchange chromatography using a HiLoad S Sepharose HP column (Pharmacia, Piscataway, NJ). Fractions containing the PEG rhG-CSF derivatives were pooled, buffer exchanged into 10 mM Na acetate, concentrated, and sterile filtered. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10–20% or 4–20% precast gradient gels (Integrated Separation Systems, Natick, MA) produced gels exhibiting single bands corresponding to apparent molecular weights of 27 and 36 kDa for the 6 kDa (P6) and 12 kDa PEG rhG-CSF (P12). The molecular weights of the PEG conjugates, determined using matrix-assisted laser desorption mass spectrometry, were 24.7 kDa (P6) and 30.7 kDa (P12). The apparent molecular weight of each PEG rhG-CSF was determined by size exclusion chromatography (SEC) using TSK-GEL G2000SW_{XL} and G4000W_{XL} gel filtration columns (Toso Haas, Montgomeryville, PA) coupled in series. Proteins were detected by UV absorbance at 280 nm (Figure 1). Bio-Rad gel filtration standards served as globu-

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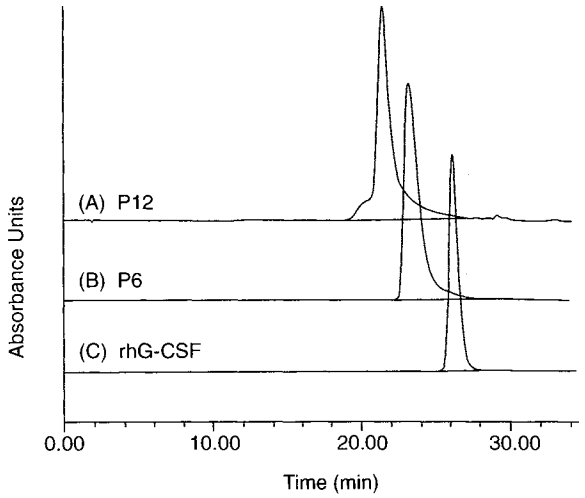

Fig. 1. Size exclusion chromatograms showing that the PEGylated proteins P12 (A) and P6 (B) elute primarily as single peaks and differ in apparent molecular weight from rhG-CSF (C). The eluting peaks were detected by UV spectrophotometry at a wavelength of 220 nm.

lar protein molecular weight markers. The apparent molecular weights obtained by this method were 53 kDa and 124 kDa for P 6 and P 12, respectively.

Cannulation and Intravenous Dosing of Rats

All rats were cannulated via the jugular vein as described before (3). Before dosing, inhalation anesthesia was induced with isoflurane and maintained with metafane from nose cones. A total dose of 25 μg/kg of rhG-CSF, 6 kDa (P6) or 12 kDa monoPEG rhG-CSF (P12) in a volume of 500 μl was injected via the penile vein. The dose of PEGylated rhG-CSF is equivalent to the amount of rhG-CSF contained in the PEGylated species. Details of the sample removal, analysis of white blood cell counts (WBC) and assay of protein are as described previously (3).

Intratracheal Instillation

This procedure was similar to that described previously (3) with one modification. After anesthetization, rats were endotracheally intubated with a small teflon catheter (The sheath only of a 1.5 in, 18G Quick-Cath; Baxter Healthcare Corp. McGaw Pk., IL) surrounded with a short piece of silastic ≈2 cm from the distal tip. This silastic acted as a seal when introduced into the trachea. Rats were instilled with rhG-CSF in a) 1 mM HCl pH ≈ 4 containing 0.01% w/v Tween 80 (≈ 75 μM) and 5% w/v sorbitol (A); b) sodium acetate buffer (10 mM Na acetate adjusted to pH 4) (B); or c) isotonic phosphate buffered saline (PBS) pH 7. (C). To determine if absorption would be influenced by volume, rats were dosed with a total of 500 μg/kg rhG-CSF contained in 100, 200, or 400 μl PBS. The PEGylated proteins were dosed IT (50 and 500 μg/kg) in buffer B. Additional experiments were conducted IT with P12 in buffer A to compare directly with the aerosol studies below.

Aerosol Dosing System

Rats were administered a general anesthetic of ketamine and xylazine as described above. They were subsequently endotracheally intubated using a Teflon catheter, and dosed with aerosol in pairs. A small animal ventilator (Harvard Apparatus, Natick, MA) was used to ventilate the rats at a tidal volume of 2.5 ml/rat and 60 beats per min. The air leaving the ventilator was passed directly into the auxiliary vent of a Devilbiss "Aerosonic" ultrasonic nebulizer containing a starting volume of 5 ml of the protein solution. The aerosol emerging from the nebulizer outlet was then passed into a 'drying' chamber containing Natrasorb T silica beads (4 × 12 mesh; Multiform Desiccants Inc., Buffalo, NY) before reaching the rats. To prevent significant heating of the protein solutions during ultrasonic nebulization, the nebulizer was modified to accommodate a heat exchanger. Refrigerant was continuously passed through the exchanger from an external water bath to prevent heating of the protein solution above 30°C during the 20-min dosing interval. The starting concentrations of rhG-CSF and 12 kDa monoPEGylated rhG-CSF were 3.2 and 2.75 mg/ml, respectively. In both instances the solutions were nebulized in buffer A.

Stability of Proteins to Ultrasonic Aerosolization

Previous work with ultrasonically nebulized lactate dehydrogenase (8) has shown that both aerosol (air-water interface) production and heating denatures the protein. In an effort to minimize surface effects, Tween 80 was added to the PEG rhG-CSF and rhG-CSF dosing solutions to give a final concentration of 0.01% w/v addition to the 5% w/v sorbitol. Solutions were ultrasonically nebulized for up to 25 min allowing normal heating of the reservoir solution or ensuring that the temperature of solution did not exceed 30°C. Samples of 100 μl were removed at intervals for analysis by SEC and PAGE as described previously (8).

Estimates of Aerosol Dose

In vitro To determine the total aerosol mass delivered rats were 'substituted' with filter units containing 47-mm glass fiber aerosol sampling filters (Gelman A/E 50, Gelman Sci., Ann Arbor, MI). The teflon catheters used to intubate the rats were inserted ≈1 cm into the inlet of the filter units but were not sealed from the atmosphere. Air flow was then drawn through each filter unit at a rate of ≈30 l/min by attaching the outlets to the house vacuum. This ensured that all aerosol emerging from the endotracheal tubes would be deposited on the filters. Solutions of rhG-CSF were doped with 2×10^{-4}% w/v carboxyfluorescein and then aerosolized for 20 min. The filters and filter unit were rinsed with fixed volumes of 50 mM NaOH. Solutions were then filtered before being assayed for the fluorochrome at excitation and emission wavelengths of 490 and 515 nm, respectively.

In vivo To 5 ml of the rhG-CSF protein solution ≈ 2.5×10^7 counts per min (cpm) of Tc-99m HSA (Syncor Inc., Van Nuys, CA) was added just prior to dosing. This was aerosolized to a total of 8 rats (4 pairs; 320 ± 11 g) for 20 min as described above. Immediately after dosing, the rats were euthanized by exposure to CO_2 and then 1 ml of blood was withdrawn by cardiac puncture and placed in a separate counting tube. The lungs and selected tissues were removed and dissected into several components (trachea, esophagus, lung lobes, lung hilus) for subsequent determination of

gamma radioactivity at 140 kEv (Cobra, Packard Instr. Co., Downers Grove, IL). From the cpm readings, estimates of the total volume of fluid reaching the lung lobes were calculated and thus estimates of the dose could be made by using the initial protein concentrations.

Data Analysis

Pharmacokinetic modeling was performed using MINSQ II (Micromath Corp, Salt Lake City, UT) as described previously (3). The rhG-CSF IV data was fitted using a biexponential model and that of the PEGylated proteins with a monoexponential model. The area under the concentration *v.* time curves (AUC) were obtained by using the log-linear trapezoid rule for IV data and a linear log-linear trapezoid combination for the pulmonary data. The area under the moment curves (AUMC) were obtained in a similar manner. The mean residence time (MRT), clearance (Cl_p) and volume of distribution at steady state (V_{SS}) were then calculated using standard techniques. To compare the WBC response, the AUC_{wbc} for each response in the interval of 0–72 h was calculated using

$$AUC^{x}_{wbc} = AUC_{wbc} - AUC^{con}_{wbc} \quad (1)$$

where the superscript, x, is the mode of delivery (IV, IT or AER) and, con represents the mean AUC_{wbc} for all control rats receiving buffer. Significance between the AUC_{wbc} values was then determined using a one-way analysis of variance (ANOVA) or by Student's t-test where appropriate.

RESULTS AND DISCUSSION

Intravenous Pharmacokinetics and Pharmacodynamics

The characteristics of the two PEGs are shown with the IV pharmacokinetic data in Table I. The apparent molecular weight of the conjugated proteins, as determined from SEC, is much larger than the unconjugated protein. However, the absolute molecular mass determined by mass spectrometry, confirms that the conjugates are monoPEGylated species with molecular weights (30.7 kDa and 24.7 kDa for the 12, and 6 kDa PEG, respectively) very close to the sum of the molecular weights of the protein (18.8 kDa) and the individual PEGs (6 k or 12 k).

The clearance of the two PEG-rhG-CSF compounds increases with decreasing molecular weight. The values are not dissimilar to previous results obtained with polyPEGylated compounds (3) where an 82 kDa PEGylated rhG-CSF exhibited a clearance rate of 0.28 ml/min/kg and a 148 kDa mainly dipegylated moiety showed a clearance rate of 0.18 ml/min/kg; slightly higher than the 0.13 ml/min/kg for P12. The distributive phase is not observed for the PEG proteins and best fits are estimated as effective $t_{1/2}$s from $MRT_{IV} * \ln(2)$ giving 107 ± 8 and 231 ± 14 min for P6 and P12, respectively. For comparison, the effective $t_{1/2}$ for rhG-CSF is 54 ± 5 min.

The WBC response to the IV administered PEG conjugates was increased relative to rhG-CSF (P12 [335 ± 113] > P6 [296 ± 111] > rhG-CSF [162 ± 120] [(WBC × 10^{-3}/μl) * h]). Although the trend in response is expected, a comparison of the AUCs according to Eq. 1 shows that only the response to the larger PEGylated protein is significantly different ($\alpha = 0.05$) from that of rhG-CSF (P12, $p < 0.05$; P6, $p < 0.08$).

Dose Estimation

The dose estimates obtained from the *in vitro* and *in vivo* data are shown in Table II. Data is presented as the dispensed volume which assumes negligible change in the concentration of the reservoir fluid during nebulization in order to convert data directly to volumes. The *in vitro* data shows no difference in the output from each of the outlets (1 and 2) suggesting that the aerosol delivered to each rat is equivalent. Some reduction in solute output results from cooling the nebulizer as has been observed previously (9). The *in vivo* deposition of radioactivity is presented as volumes (μl) and in the form of an evenness index (EI) for the deposition in the lung lobes and bronchi as described by Brain and colleagues (5)

$$EI = \frac{\left(\frac{\text{cpm}}{\text{wet weight (tissue)}}\right)}{\left(\frac{\text{cpm}}{\text{wet weight (lung)}}\right)} \quad (2)$$

A value of one indicates homogeneity. The results illustrate that deposition in the lung lobes is relatively homogeneous. The large excess of radioactivity deposited in the trachea is possibly due to condensation of droplets at the exit of the endotracheal tube. Hypersecretion of mucus in the upper airways and trachea in response to the presence of the catheter may contribute to the increased deposition in this region by narrowing the airway and acting as an additional impaction site. The approximately 5-fold difference in the deposited (*in vivo*) *v.* dispensed (*in vitro*) volumes probably reflects the deposition efficiency during tidal breathing in the rats.

Stability of Proteins to Ultrasonic Aerosolization

The rhG-CSF in buffer solution A is unaffected by ul-

Table I. Pharmacokinetic Data Obtained After Intravenous Dosing of rhG-CSF and PEGylated G-CSF

Treatment group	Apparent MW (kDa)[a]	n	Dose (μg/kg)	MRT (min)	Cl_p (ml/min/kg)	V_{ss} (ml/kg)
P12	124	7	24.9 ± 0.5	333 ± 20	0.13 ± 0.01	42.2 ± 1.1
P6	53	5	24.2 ± 0.6	155 ± 5	0.29 ± 0.02	44.5 ± 2.7
rhG-CSF	19	6	26.3 ± 1.1	76.5 ± 8	0.81 ± 0.17	61.7 ± 4.6

[a] Apparent molecular weight (MW) determined from size exclusion chromatography data. Mass spectrometry. Molecular masses are 30.7 and 24.7 kDa for P12 and P6, respectively.

Table II. *In Vivo* and *in Vitro* Estimation of Aerosol Deposition

	Volume[a] (μl)	Evenness index[b]
In vivo		
Trachea	15 ± 6.3	—
Bronchi (hilus)	2.9 ± 1.3	1.03 ± 0.20
Left lung	2.0 ± 0.6	1.06 ± 0.05
Right upper	0.8 ± 0.3	1.05 ± 0.23
Right middle	0.6 ± 0.2	1.04 ± 0.26
Right lower	1.5 ± 0.5	0.97 ± 0.12
Mediastinal	0.6 ± 0.2	0.74 ± 0.12
Blood[c]	4.5 ± 1.3	—
Other[d]	4.0 ± 2.4	—
Total lung lobes	12.9 ± 3.4	—
Total lung	31.9 ± 10.5	—
In vitro[e]		
Total filter 1—cool	189 ± 36	
Total filter 2—cool	195 ± 48	
Total filter 1—norm	257 ± 42	
Total filter 2—norm	254 ± 39	

[a] Calculated from deposited radioactivity or recovery of fluorochrome from filter unit (see text). Data shown are the mean ± SEM (n = 8).

[b] Evenness index calculated using Eq. 2.

[c] Blood component added to lung lobe total. Total in blood assumes blood volume equivalent to 7% body weight.

[d] Includes esophagus and residue from dissection.

[e] Nebulizer modified (cool) to maintain operation at below 30°C or allowed to operate normally (norm). Data shown are the mean ± SD (n = 3).

trasonic nebulization. No changes in shape or area of peaks eluting from SEC result and no additional bands are seen on SDS- and native-PAGE gels. In contrast, the 12 k PEG rhG-CSF is aggregated by ultrasonic nebulization when heating of the protein solution is allowed (±Tween 80). When solutions are maintained at <30°C no aggregation is observed. These results suggest that the aggregation caused by ultrasonic nebulization is related to the melt temperature (T_m) of the respective proteins. T_m values were subsequently determined by Peltier heating of samples while monitoring the absorbance at 340 nm. The T_m for the PEG rhG-CSF by this method is 63°C but no increase in light scattering of the rhG-CSF is noted even when heated to 90°C. This suggests that the rhG-CSF does not aggregate above its melt temperature and this helps explain the results observed during ultrasonic nebulization. Heating curves have previously been obtained for "Aerosonic" nebulizer solutions (9) and the temperature within the ultrasonic fountain can exceed 80°C. Hence, aggregation of the PEG rhG-CSF can be expected during nebulization but as noted, aggregation of rhG-CSF is unlikely to occur in response to heating alone.

Intratracheal Instillation and Aerosol Administration

Striking differences exist between the systemic protein concentration *v.* time curves for the instilled *v.* aerosolized proteins (Figures 2 A and B). The rhG-CSF is apparently absorbed rapidly when aerosolized to the lungs. Significant levels of protein are present in the circulation within minutes of initiating aerosolization. Peak plasma levels are reached 21.7 ± 4.8 minutes after halting aerosolization (Table III). The apparent bioavailability after aerosol administration, based on estimates of dose reaching the lung probes (F_{lung}), is 66 ± 14% (n = 8). In comparison, the F_{lung} values for the proteins administered by IT in buffer A at 500 μg/kg are 11.9 ± 1.5% (n = 3). These differences are significant ($p < 0.05$). A similar scenario applies to the 12 k PEG rhG-CSF where the F_{lung} values are increased from 1.6 ± 0.1% by IT to 12.3 ± 1.9% after aerosol administration ($p < 0.01$). The differences in extent of absorption between P12 and rhG-CSF are likely related to differences in the molecular dimensions of the two proteins. It is difficult to grasp exactly what the 'size' will be when the proteins are presented to the alveolar epithelium in the lung microenvironment, but the information from SEC gives some indication that the apparent molecular mass of the PEGylated protein in solution is significantly larger than its true mass when referenced against a series of globular proteins of known molecular weight.

Since the C_{max} values are reached quickly after aerosol dosing there is a higher likelihood that the post-peak phases will be less contaminated with the absorptive phase of protein than after intratracheal instillation. Consequently, any change in the slope of the post-peak phase relative to the IV slopes should be indicative of changes that have occurred to the proteins after deposition in the lung. Neither the rhG-CSF nor the P12 appear to have been adversely affected by absorption.

The results are in general agreement with other investigations that have contrasted aerosol and intratracheal instillation. Colthorpe and coworkers (10) have shown a 5-fold

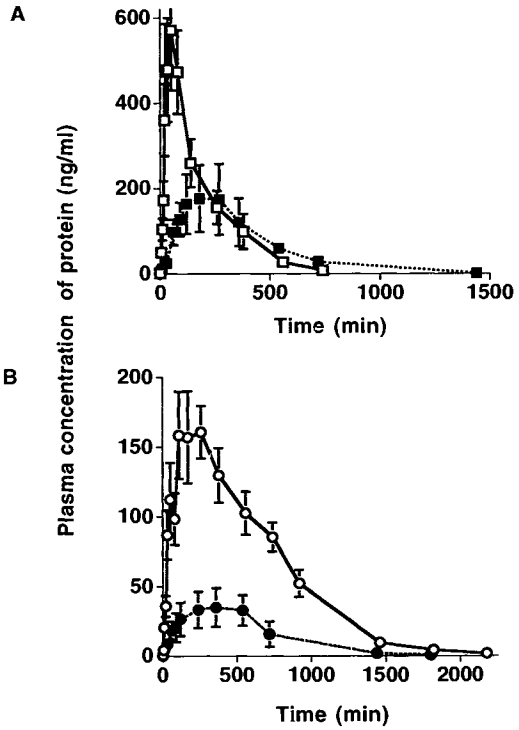

Fig. 2. Plasma protein concentration *v.* time curves after aerosol and IT administration of rhG-CSF (A) and P12 (B). Aerosol administration of the rhG-CSF (□) and P12 (■) results in more rapid absorption and greater overall availability (Table III) than achieved after IT administration of rhG-CSF (○) and P12 (●).

Table III. Pharmacokinetic Data Obtained After Aerosol Administration of rhG-CSF and PEGylated rhG-CSF[a]

Treatment group	n	Dose (μg/kg)	MRT (min)	F_{lung}[b] (%)	T_{max} (min)	C_{max} (ng/ml)
P12	7	110 ± 29	531 ± 32	12.3 ± 1.9	168 ± 31	182 ± 14
rhG-CSF	4	129 ± 34	192 ± 16	65.9 ± 14	21.7 ± 4.8	598 ± 135

[a] Solution of protein in 5% w/v sorbitol, 0.01% w/v Tween 80, pH ≈ 4.
[b] Based upon estimated dose of Tc-99m HSA deposited in the lung lobes.

increase in the extent of insulin absorption after aerosol delivery. Schanker and colleagues (11) have demonstrated that the *rate* and extent of absorption can be improved after aerosol delivery and recently Colthorpe and colleagues (12) have been able to demonstrate this with growth hormone. Both insulin and growth hormone exhibit absorption rate limited pharmacokinetics and this allows the apparent absorption rate constants to be directly estimated from the slopes of the post-peak absorption curves. Unfortunately, the post-absorptive phases of the rhG-CSF curves are dependent upon the elimination rate from the circulation and further analysis of concentration *v.* time data is difficult.

Other factors to consider in the absorption of proteins include surface activity; a characteristic of many proteins. For example, a 1 mg/ml aqueous solution of rhG-CSF in 1 mM HCl gives an equilibrium surface tension of ≈ 48 mN/m. The addition of surfactant to solutions has been shown to enhance the spread of instilled solutions of technetium colloid and pentamidine in the lung (13). Thus, this physicochemical characteristic may improve the overall deposition and potentially the absorption of proteins. The stability of the protein in the lung environment will also be important. If degraded rapidly, the amount of intact protein appearing in the circulation will be dependent upon the relative rates of absorption and degradation. rhG-CSF is known to be unstable in an isotonic salt environment although the destabilization rate is slow relative to the appearance of the protein in the circulation. Additionally, rhG-CSF is known to interact and remain stable within lipid bilayers (14): a factor that also may influence uptake.

A further question to raise is whether transport mechanisms for specific cytokines exist in the lungs. Dranoff and coworkers (15) have demonstrated that transgenic mice deficient in the gene encoding GM-CSF *only* exhibit a disorder in pulmonary homeostasis suggesting that there is a reason for this 'hematopoietic' protein to be present in the lung. It is also known that alveolar macrophages release various cytokines, that bronchial epithelial cell lines can secrete G-CSF (16) and alveolar type II cell-lines can release cytokine receptors in response to viral infections (17). Considered together, this evidence indicates a complex interplay of signaling between cells in and out of the lungs and it is likely that pathways are present to transport cytokines back and forth across cells. However, as a word of caution when attempting to associate absorption with a physiological mechanism, the above experimental observations have been made under distinctly nonphysiological conditions. The amount of protein administered far exceeds 'normal' physiological levels. Hence, any transport mechanism specific for a macromolecule may be overwhelmed causing absorption to occur by any available route or by a route that is artificially created to deal with the anomalous presence of a 'recognized' protein.

The WBC response to the aerosolized and instilled proteins (500 μg/kg) dose is shown in Figures 3A and B. The response to the 12 kDa PEGylated protein was not significantly extended relative to that of rhG-CSF after either IT (AUC: 641 ± 154 rhG-CSF; 649± 309 P12, p = NS) or aerosol dosing (AUC: 383 ± 200 rhG-CSF; 264 ± 132, p = NS) in units of (WBC × 10^{-3}/μl) *h). This is despite a longer circulating half-life. This may be a reflection of the extent of absorption in both instances, the result being that the available doses are not comparable. The WBC responses to all the instilled proteins at both doses were also not significantly different from each other as a group.

The Influence of Buffer and Volume After IT Dosing

The aerosol and IT experiments were conducted with

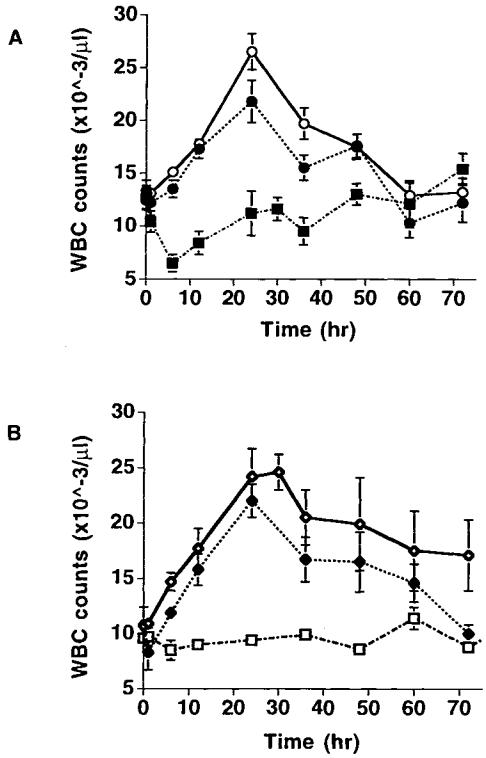

Fig. 3. The WBC response to aerosol and IT administration of 500 μg/kg rhG-CSF and P12. The overall response to the rhG-CSF (A) is not significantly different for aerosol (●) and IT (○). Similarly, the response to P12 does not differ by either route (Aerosol (♦); IT (◇)). However, the responses are greater than observed with controls receiving buffer by IT (□) or by aerosol (■).

Table IV. The Influence of Intratracheally Instilled Buffer[a] and Instilled Volume on the Pulmonary Pharmacokinetics of rhG-CSF and PEGylated rhG-CSF

Treatment group[a]	n	Dose[b] (μg/kg)	MRT (min)	F_{lung}[c] (%)	T_{max} (min)	C_{max} (ng/ml)
rhG-CSF						
A	3	[500] 559 ± 35	427 ± 30	12 ± 1.5	100 ± 17	105 ± 12
B	3	[50] 45.0 ± 1.4	244 ± 11	3.0 ± 0.5	110 ± 26	3.6 ± 0.6
B	6	[500] 524 ± 23	277 ± 15	16 ± 5.1	160 ± 27	108 ± 24
C	5	[50] 54.3 ± 0.9	252 ± 19	7.3 ± 1.0	132 ± 19	11.0 ± 1.1
C 250 μl	6	[500] 421 ± 10	321 ± 12	13 ± 3.4	210 ± 30	100 ± 13
C 100 μl	6	[500] 509 ± 30	252 ± 20	7.1 ± 1.3	115 ± 14	85 ± 11
C 400 μl	6	[500] 429 ± 8	289 ± 16	8.2 ± 1.6	150 ± 13	77 ± 11
P12						
A	3	[500] 543 ± 21	560 ± 56	1.6 ± 0.1	310 ± 121	65.9 ± 14
B	5	[50] 47.1 ± 0.9	506 ± 40	1.2 ± 0.8	473 ± 55	7.5 ± 2.3
B	5	[500] 439 ± 17	500 ± 15	1.2 ± 0.7	375 ± 51	38.6 ± 6.1
P6						
B	6	[500] 415 ± 11	578 ± 61	4.5 ± 2.0	270 ± 27	66.8 ± 20
B	6	[50] 48.7 ± 1.3	607 ± 78	3.3 ± 0.7	428 ± 46	8.1 ± 1.6

[a] A = 1 mM HCl + 0.01% w/v Tween 80 and 5% w/v sorbitol; B = 10 mM sodium acetate adjusted to pH 4; C = phosphate buffered saline (pH 7). The nominal volume of solution dosed to the animals was 250 μl unless stated otherwise. All data shown is the mean ± SD.

[b] Values in brackets represent the nominal dose.

[c] Estimate of dose based upon delivery of Tc-99m HSA deposited in the lung lobes.

solutions of protein in a pH 4 buffer containing surfactant. The reason for the rapid absorption may have been due to the pH and/or the surfactant: both could influence absorption by damaging the pulmonary barrier. The proteins could not be aerosolized using different buffers at neutral pH without causing substantial aggregation. Therefore, additional experiments were completed by IT using a neutral pH buffer where a stock solution of 4 mg/ml rhG-CSF was admixed with PBS (pH 7) just before dosing (*C*). The resultant F_{lung} values do not show any significant difference from that of the lower pH and surfactant solutions (Table IV). Nevertheless, there is ample evidence that low pH aerosols can damage lung tissue (18). In addition, Komada and others (19) have shown that an isotonic pH 3 citrate buffer increases the absorption of several small proteins relative to a pH 5.5 buffer. Similarly, Smith and colleagues (20) have shown that pH 2.5 solutions increase the rate but not the extent of absorption of an RGD peptide. One simple explanation for our observations is that the dosing solutions are minimally buffered at pH 4 and hence have low titratable acidity. Upon contact with the lung fluids, solutions are presumably rapidly buffered to the pH of the local environment since the mucus is known to possess a relatively high buffer capacity (21).

Given the surface-active environment into which the protein is being delivered and the fact that rhG-CSF has significant surface activity itself it seems unlikely that low concentrations of Tween 80 (≈ 75 μM) will enhance absorption. Even 20 mM concentrations of the surfactant at similar dosing volumes have been shown to only marginally improve the pulmonary absorption of insulin (22). If increased absorption were dramatic, it probably would be associated with epithelial damage and inflammation caused by properties of the surfactant that are not directly related to surface activity.

Finally, to confirm that the dosing volume was not influencing absorption and thus contributing to the variability associated with IT dosing, fixed doses of 500 μg/kg rhG-CSF in volumes of 100, 250, and 400 μl were administered to the lungs. Again, no changes in absorption were observed ($p < 0.05$; ANOVA) using this volume range (Table IV).

CONCLUSIONS

These experiments demonstrate that the extent of absorption of rhG-CSF and monoPEGylated rhG-CSF is significantly greater after aerosol administration than after intratracheal instillation. The evidence also suggests that the proteins are absorbed intact and retain biological activity. Additionally, Tween 80 at low concentrations and unbuffered solutions of pH 4 do not appear to influence the extent or nature of absorption of the rhG-CSF after intratracheal instillation. To develop this work further, efforts are needed to establish the absorption sites and to determine if the absorptive pathways are similar for the PEGylated and nonPEGylated proteins.

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