Pulmonary Delivery of Powders and Solutions Containing Recombinant Human Granulocyte Colony-Stimulating Factor (rhG-CSF) to the Rabbit

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Two powder formulations (MMAD <4 μm) containing rhG-CSF were insufflated (IF) via an endotracheal tube at doses of 5, 75 or 500 μg/kg to New Zealand white rabbits. Doses of 5 and 500 μg/kg of solutions were administered by intratracheal instillation (IT), subcutaneous (SC) injection in the thigh and intravenous injection (IV) via the marginal ear vein. Blood samples were removed at regular intervals from an indwelling jugular catheter. Blood was analyzed directly for total white blood cell counts (WBC). Plasma was assayed for rhG-CSF by a specific ELISA. The distribution of radioactive dose in lung tissue was found after administering Tc99m HSA in solution or when incorporated into powders. The pharmacokinetics and pharmacodynamics were determined for all routes of administration. High dose IV concentration vs. time profiles declined biexponentially ($t_{1/2}\alpha$ = 0.6 ± 0.2 hrs, $t_{1/2}\beta$ = 4.6 ± 0.2 hrs, n = 8). Clearance was dose dependent (11.6 ± 2.6 [500 μg/kg, n = 8] vs. 21.8 ± 3.3 ml/hr/kg [5 μg/kg, n = 5]). A normal systemic response was obtained after IF, indicating that rhG-CSF retains activity in the solid state. Dissolution and absorption of rhG-CSF from the powders were not rate limiting. The plasma concentration vs. time profiles peaked at similar times to those after IT (T_{max} 1–2 hrs) but were earlier than obtained after SC (T_{max} 6–10 hrs). Powders were less efficiently dosed to the lung lobes after insufflation compared with instillates (14.7 ± 10.5 vs. 60.1 ± 10.6%), resulting in bioavailabilities ranging from 5 to 33%. Bioavailability after SC was 11.0 ± 7.0% and 95.3 ± 7.9% (n = 6) for the low and high doses, respectively.

KEY WORDS: rhG-CSF; intratracheal instillation; lung; pharmacokinetics; pharmacodynamics; pulmonary absorption; Tc-99m.

INTRODUCTION

The therapeutic use of rhG-CSF stems from the need for neutrophilic granulocytes in a response to infection. An absence or significant lowering of circulating neutrophils predisposes toward infection (1). In response to a bacterial infection, circulating levels of natural G-CSF are increased and blood granulocyte levels in humans are normally elevated 3 to 5× from baseline (2). Administration of rhG-CSF at an appropriate dose level can readily induce a similar neutrophilia (3). Currently, the *E. coli* derived product, filgrastim, is used to decrease the incidence of infection as manifested by febrile neutropenia, in patients receiving myelosuppressive anti-cancer drugs, and in severe congenital neutropenia. Clinical trials are underway for a number of other related indications. Subcutaneous injection or intravenous infusion are the current preferred routes of administration but given the variety of patient populations in which rhG-CSF is clinically indicated it is possible that some patients would prefer using a noninvasive route of administration. A recent article by Machida et al. (4) has shown that the nasal route can be utilized to administer the CHO derived, rhG-CSF. However, the pharmacological activity was only 5 to 10% of that achieved by subcutaneous injection (4). This was improved up to 3× by using surface active agents as penetration enhancers. Pulmonary delivery via inhalation has also received some attention and it has been shown that aerosol administration (5) and intratracheal instillation of rhG-CSF in hamsters (6) can induce a pharmacological response that is dose-dependent. It was estimated that ≈62% of an instilled dose reached the circulation. Absorption of the protein from the lung was apparently rapid and this process was not the rate-determining step in clearance from the circulation (6). These observations have prompted further work and in this study we examine the feasibility of administering rhG-CSF as a dry powder. The pharmacokinetics and pharmacodynamics of single doses of rhG-CSF delivered as an instilled solution or insufflated powder to the lungs of rabbits. The results are compared with those of a single intravenous or subcutaneous injection.

MATERIALS AND METHODS

Preparation and characterization of rhG-CSF powders and solutions

rhG-CSF was obtained from Amgen manufacturing, lot #T6705 at a concentration of 4.04 mg/ml in unbuffered 1 mM HCL. In this environment the protein exists as a single chain polypeptide of 18.8 kDa containing 2 disulfide bridges both of which are important for retention of biological activity. Dilution of the stock rhG-CSF was performed with double distilled water. The diluted solutions were used within 12 hr of preparation.

The physico-chemical stability of the rhG-CSF in powders and solutions was monitored by polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography (SEC). The latter was performed using a 1% agarose column (Superose 12, Pharmacia LKB Biotechnology, Piscataway, NJ) with a mobile phase of 0.1 M $NaHPO_4$ running at 0.5 m/min. Powders were reconstituted in solutions of 1 mM HCL before analysis. The *in vitro* bioactivity was determined in a mitogenic cell culture assay. The uptake of tritiated thymidine in response to rhG-CSF was monitored in 32DCL3 cells.

Two powders were prepared by spray drying. One consisted of pure rhG-CSF (G), the other contained a coprecipitate of 1 part rhG-CSF to 5.6 parts excipients (G+). Once collected and characterized, the powders were sealed under vacuum, over desiccant at room temperature until used. The mass of powder insufflated to the rabbits was fixed at 500 μg/kg ie ≈1.5 mg/animal. Higher amounts of powder were difficult to dose effectively using the technique

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described below. The fixed powder dose also required that the prospective 5 μg/kg doses be homogeneously mixed with a bulking agent consisting of mannitol of similar particle size prior to dosing. Similarly, the nominal high dose of G+ had to be reduced to 75 μg/kg to retain a total powder dose of ≈1.5 mg. This was accomplished by mixing with spray-dried mannitol of similar particle size. The mixing and transfer of powders to the dosing tubes were performed in a dry box maintained at <5% relative humidity at room temperature.

The particle size of each powder was checked by photosedimentation centrifugation (CAPA-300; Horiba Inc., Irvine, CA), time of flight aerosol beam spectrometry, (TOFABS; Aerosizer Mach II, API, Amherst, MA) and scanning electron microscopy (SEM; Jeol JSM 5200, Jeol Inc., Peabody, MA). The density correction used with TOFABS was 1 for the protein and 1.49 where sugars were used. The methodology for these techniques has also been discussed elsewhere (7).

Cannulation of rabbits

Female New Zealand white rabbits (Western Oregon Rabbit Co., Philomath, OR) were quarantined for at least a week after receipt. Animals were allowed to feed normally pre- and postsurgery. Rabbits were housed and studied according to the guidelines documented by the US department of health and human services (8).

A silastic based catheter was prepared that was modified from the design of Walsh et al. (18). A 0.25 in length cuff of polyethylene tubing (PE 280; Intramedic polyethylene tubing, Clay Adams, Parsippany, NJ) was slipped 6.5 cm over the end of a ≈26 cm length of silastic tubing (0.04 in id, 0.085 in od) and secured in place using super glue. The assembly was subsequently sterilized by gamma-irradiation.

General anesthesia in the rabbits was induced by intramuscular injection of ketamine (40–50 mg/kg) and xylazine (5–10 mg/kg) into the dorsal thigh. The xylazine was administered 10 min. before the ketamine. After surgery, 40 ml of lactated-Ringer's solution was injected SC into the lower back (20 ml either side).

The silastic catheter was placed into the jugular vein using an operative technique modified from that of Walsh et al. (9). Briefly, A 2 cm incision, running head to toe, was made in the right anterolateral cervical region about 3 cm below the angle of the jaw. A segment of the external jugular vein was carefully exposed just below the bifurcation of the internal and external maxillary veins. The silastic cannula was fed in toward the heart, via an incision in the vein, until the cuff was reached. The inserted portion of the cannula was tied in place with 2 ligatures of 4-0 silk. A further ligature was placed around the cuff to prevent slippage of the cannula from the vein. Two further ties were placed around the external portion of the cannula and the internal maxillary vein. The remaining proximal portion of the cannula was tunneled subcutaneously and exited through the skin at the interscapular region of the back. The cannula was taped in place and flushed with heparinized saline (30 Units/ml) and sealed with a short stainless steel pin. The neck incision was cleaned with alcohol and closed with 3-0 silk sutures. These were supported by wound clips. Pharmacokinetic studies were initiated 24 hrs post-cannulation.

Dosing of rabbits

Rabbits were administered rhG-CSF by one of the following routes: intravenous injection (IV), subcutaneous injection (SC), intratracheal instillation (IT) or intratracheal insufflation (IF). Irrespective of the route of administration, all animals were anesthetized before dosing with ketamine (40–50 mg/kg) and xylazine (5–10 mg/kg).

For IV dosing a 23 ga. butterfly cannula (12 in. tubing infusion set, Abbott Inc., North Chicago, IL) was inserted into the marginal ear vein. Animals were then injected with ≈0.75 ml of accurately weighed rhG-CSF solution. After injection, the dose was flushed into the vein with sterile saline. For SC dosing ≈2 ml of rhG-CSF solution was injected into a shaved area of skin of the lower back.

To complete the intratracheal dosing, the anesthetized rabbits were straddled, face up, on a slant board at 60 deg. The animals were supported from a wire, connected to the board, running underneath their front incisors. A pediatric uncuffed endotracheal tube (3.0 mm id, 4.3 mm od, 16 cm length [without adapter]; Mallinckrodt critical care, Glen Falls NY) was placed into the trachea. Intratracheal instillation was performed by a similar general method to that described previously for hamsters (6). In this instance 0.5 ml of dosing solution was instilled from a 1 ml syringe via a 20 ga. pipetting needle with a 15 cm barrel (Popper and Sons Inc., New Hyde Park, NY). The needle was sheathed with another tube connected to a 20 ml syringe and placed into the endotracheal tube to the hub of the syringe. Air and instillate were administered simultaneously by depressing both syringe barrels together. Post-mortem examination of a rabbit with the dosing mechanism in place, revealed that the position of the endotracheal tube extended ≈1.5 in beyond the larynx and was 3 in short of the tracheal bifurcation. The tip of the dosing needle extended to 5 mm from the distal end of the endotracheal tube when tested *in vitro*.

Dispersion of a quantitative dose of powder into the lung was accomplished by utilizing the delivery technique shown in Fig 1. This involved placement of a single dose of accurately weighed powder, "C" into the end of an elongated microvolume pipet tip, "D" (Microflex pipet tip; National Scientific Supply Co. San Rafael, CA). The tip was then rolled between the fingers to ensure the powder was lightly packed toward the distal opening. The inside diameter at the exit was 0.3 mm. The opposite end of the tip was partially sheathed with a short ≈1 cm piece of 4.8 mm id, 6.4 mm od Tygon tubing "B." About 5 mm was left extending over the edge of the tip. This provided an air-tight seal when screwed into a molded Luer lock at the end of a 20 ml plastic syringe "A" (Becton Dickinson Inc., Franklin Lakes, NJ). The assembly was connected to a 12 cm length of flexible Teflon tubing with an id and od of 1.07 and 1.9 mm respectively (Small Parts Inc., Miami Lakes, FL). The extra length protected the opening of the tip from moisture inside the respiratory tract while it provided enough length for the whole assembly to reach close to the distal end of the endotracheal tube, "E" when in position for dosing. The volume of air in this extension tube also helped to blow any moisture out of the end before any powder dose followed. To dose, the extended syringe barrel was rapidly depressed to deaggregate and force the drug from the pipette tip into the lung.

Table I. Rabbit Dose Data

Treatment group	n	Dose weight[a] (mg)	Animal weight (kg)	Dose G-CSF (μg/kg)
500 μg/kg				
Intravenous Inj.	8	800 ± 72	3.1 ± 0.2	535 ± 35
Subcutaneous inj.	6	2092 ± 35	3.0 ± 0.1	535 ± 44
Solution instillation	6	586 ± 66	2.9 ± 0.1	474 ± 62
G powder insufflation	5	1.4 ± 0.8	3.2 ± 0.2	595 ± 126
G+ powder insufflation[b]	6	1.8 ± 0.2	3.0 ± 0.1	94 ± 11
5 μg/kg				
Intravenous inj.	5	826 ± 76	3.2 ± 0.3	5.2 ± 0.2
Subcutaneous inj.	6	2084 ± 60	3.0 ± 0.1	5.3 ± 0.2
Solution instillation	6	678 ± 35	2.9 ± 0.1	6.2 ± 0.4
G powder insufflation	6	2.3 ± 0.9	3.2 ± 0.2	10.3 ± 4.1
G+ powder insufflation	6	2.2 ± 0.5	3.0 ± 0.1	9.2 ± 2.4
Controls				
Intravenous inj.	3	799 ± 26	3.2 ± 0.2	—
Subcutaneous inj.	3	2051 ± 29	3.0 ± 0.1	—
Solution instillation	3	629 ± 53	3.1 ± 0.3	—
Powder insufflation	3	1.8 ± 0.7	3.2 ± 0.1	—

[a] Solution doses refer to weight of fluid dispensed from dosing syringe.
[b] The nominal mass of powder to be dosed to the animals was fixed at 1.5 mg. Since the G+ powder was a coprecipitate it was not possible to dose 500 μg/kg rhG-CSF without exceeding the mass limit. The nominal dose was therefore reduced to 75 μg/kg to account for this.

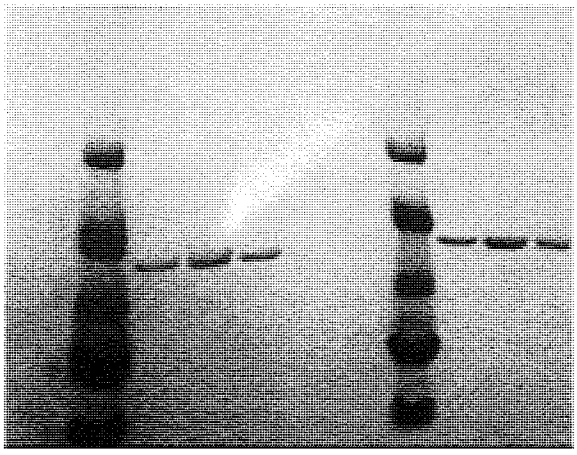
tration at the last time point and β is the terminal slope. The mean residence time (MRT) was obtained from AUMC/AUC and the maximum plasma concentration (C_{max}) and its time (T_{max}) was taken from the observed data. Rate constants were determined using a nonlinear least squares regression program, MINSQ II. The IV kinetics were described by a biexponential function. The volume of distribution of the central compartment (V_c) was estimated from the IV dose/(A + B) where A and B are the coefficients of the biexponential equation. The weighting factor used was 1/(standard deviation).

RESULTS AND DISCUSSION

Preparation and characterization of rhG-CSF powders and solutions

The *in vitro* bioactivity of lot #T6705 was 0.72×10^8 U/mg. This value falls within the normal specification limits of $1 \pm 0.6 \times 10^8$ U/mg. The bioactivity for the G and G+ powders estimated after completion of the study fell within specification. The stability of rhG-CSF assessed by SDS-PAGE is shown in Fig. 2. In all cases, before and after completion of the *in vivo* studies a single band on a coomassie-blue stained gel is apparent which is consistent with the control rhG-CSF. SEC also demonstrated that >95% of the integrated peak area was associated with the monomer protein.

All powders had volume and mass median diameters less than 3 μm as determined by TOFABS and photosedimentation. The powder G was sized at 2.5 μm MMAD ± 1.6 GSD and 2.3 μm VMD and G+ 2.7 μm ± 1.7 and 2.3 μm for the two instruments respectively. In turn, the mannitol was sized at 3.4 ± 1.4 and 3.7 μm. A scanning electron micrograph illustrates the non-spherical nature of the particles produced by the spray drying method in the presence of protein (Fig 3). The powders were white with a low apparent bulk density due, in part, to the presence of loose aggregates or flocs.



soln = rhG-CSF solution standard T6705

Fig 2 A SDS gel stained with coomassie blue showing the absence of any bands indicating aggregation in both rhG-CSF powder formulations after reconstitution in 1 mM HCL. From left to right, under non-reducing conditions, the wells are a) molecular weight standards, b) G-CSF solution standard T6705, c) powder G and d) powder G+. This order is repeated in additional wells after reducing samples with β-mercaptoethanol.

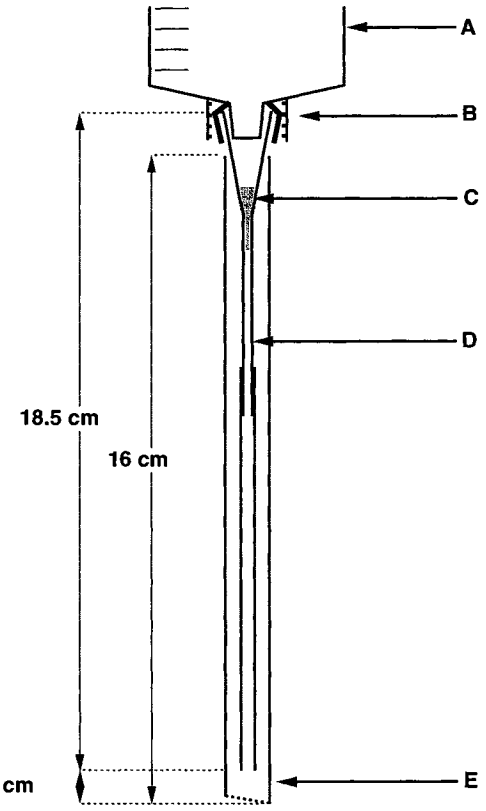


Fig 1 The mechanism used to insufflate powders to the rabbit lung. A dosing tube "D" was carefully filled with 1.5 mg of accurately weighed powder formulation "C". A 20 ml syringe, A, was then connected via the luer lock to an elongated dosing tube "D" sheathed with a 1 cm length of flexible tubing "B" to provide an air-tight seal. The dosing tube was then inserted through a 3 mm id pediatric endotracheal tube, "E" as far as it would extend. The endotracheal tube was positioned in a previously intubated rabbit.

To gauge the performance of the method *in vitro,* the dosing tube was first filled with ≈1.5 mg of the G powder. The complete mechanism was positioned vertically above the entrance of a cascade impactor (Andersen Inc., Atlanta, GA) with the opening of the endotracheal tube level with the entrance to the impactor preseparator. The dosing tube was actuated after starting the impactor. This process was repeated 5× to collect sufficient powder on the impactor stages for analysis. The rhG-CSF was then washed off the plates with 3 ml of 1 mM HCL and quantified directly by UV spectrophotometry (HP 8452 Diode array spectrophotometer, Hewlett Packard Co. North Hollywood, CA) at a wavelength of 280 nm.

Determination of dosing efficiency to the lung

To assess the distribution of dose in the rabbit lung, a radioactive marker of Technetium-99m labeled human plasma albumin (Tc-99m HSA, USP XXI 1985 p1012) was added to powder and solutions of G-CSF. 1 ml of 1 mCi of the Tc-99m HSA was obtained from a commercial source (Syncor Corp., Van Nuys, CA). It's radio-chemical purity was checked by running a 2.5 ml sample of diluted stock solution through a sephadex G-25M PD-10 column (Pharmacia LKB Biotechnology, Piscataway, NJ) with 15 ml saline. Fractions of 0.5 ml were collected and analyzed on a gamma counter (Cobra Auto-Gamma, Packard Inst. Co. Downers Gr., IL). For intratracheal instillation, dosing solutions containing ≈500,000 cpm were prepared with 0.5% w/v bovine plasma albumin (BSA; Fraction V, Sigma, St. Louis, MO) added to minimize loss of radio-label to the syringe walls. To prepare the radio-labeled powder, a sufficient volume of the stock solution was added to the spray-drying solution to produce a final powder that contained ≈500,000 cpm/1.5 mg dose at the time of dosing. Two groups of 5 rabbits were dosed with either powder or solution using the techniques described above. Immediately after dosing, the animals were sacrificed and the lungs were removed. It was assumed that the HSA would act as a nonabsorbable marker and that losses due to absorption between the time of dosing and dissection of the lung were negligible. The lungs were dissected into their lobar components, (left upper, left lower, right upper, right middle, right lower, and mediastinal) the trachea, and esophagus, were separated. The mouth, was also washed out with 5 ml of saline. Samples were placed in polyethylene gamma counter tubes (1 cm diameter × 10 cm length) and radioactivity was detected using a gamma counter.

Time course studies

Rabbits were divided into treatment groups of six. All groups were administered nominal doses of 5 or 500 μg/kg rhG-CSF except one that received 75 μg/kg of rhG-CSF as the G+ powder. Control groups of 3 rabbits received the vehicle or excipients associated with the mode of delivery. The dosing data for each group is shown in Table I. Baseline blood samples of 1 ml were removed from the animals before dosing. Blood samples of ≈0.75 ml of blood were then taken at the following times: 1, 5, 10, 15, 20, 30, 45, 60 min, 1.5, 2, 3, 4.5, 6, 9, 12, 21, 24, 36, 48 and 72 hrs. In some instances, where the white blood cell response (WBC) did not return to baseline in the 3-day period, sampling was continued on a daily basis until baseline was reached. Circulating white blood cell (WBC) counts were determined using an automated blood cell analyzer (Sysmex F800, Microcell counter, Toa Medical Electronic Co. Kobe, Japan). For differentials, 3 μl blood smears were prepared on slides and subsequently treated with Wright-Giemsa stain prior to microscope differentiation of cells. Plasma was obtained after centrifuging the blood for 10 mins at 10000g. This was stored, azide free, at −70°C until assayed for the presence of rhG-CSF using an enzyme linked immunosorbent assay (ELISA) (R&D systems, Minneapolis, MN). Calibration curves were constructed using the Amgen rhG-CSF lot #T6705 as the standard.

Pharmacokinetic analysis

The pharmacokinetic parameters of clearance (Cl_s), volume of distribution at steady state (V_{ss}) and the bioavailability based on the dose leaving the syringe (F) and the dose reaching the lung lobes (F_{lung}) were calculated from the area under the curve vs. time (AUC) and the area under the curve × time vs. time (AUMC) profiles using standard methods (10). The linear trapezoid rule was used to determine the AUC and AUMC values. These values were extrapolated to infinity using C#/β where C# is the plasma G-CSF concen-

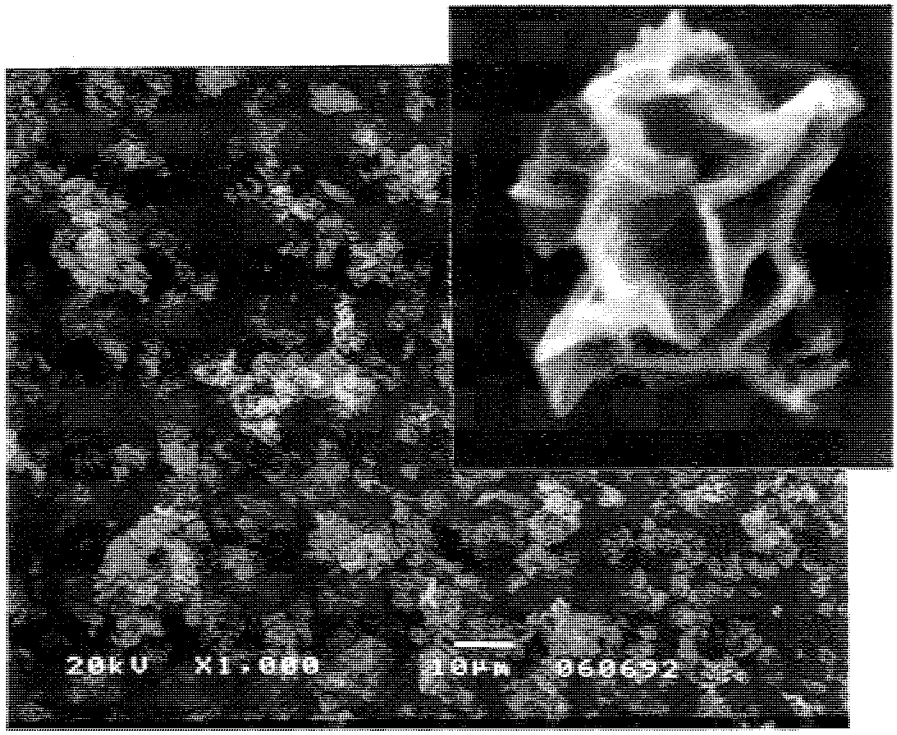


Fig 3 A scanning electron micrograph of an rhG-CSF powder formulation at 1000× magnification and 20 KeV. The insert shows a 20000× magnification of a single particle. This illustrates the rugose surface of the particles containing protein. The particulates were dispersed onto the stub using the same mechanism described to dose the animals.

Dosing of rabbits and determination of dosing efficiency to the lung

The radiochemical purity of the Tc-99m-HSA was determined to be 98.0% by column chromatography. The fraction of the recovered radioactivity reaching the lung lobes was significantly different between the powder and solution doses ($p < 0.0011$). Only 14.7 ± 10.5% (27.9 ± 17.9%) of the administered (recovered) powder dose reached the lung lobes compared with 60.1 ± 10.6% (79.2 ± 5.3%) of the instillate. The majority of powder appears to have deposited in the trachea as it emerged from the extension tube. This deposition profile is also suggested by the *in vitro* results generated using the cascade impactor where 51.7% of the dose was recovered from the preseparator (>10 μm) while 31.8% was less than 5 μm. Within the lungs, the gas forced through the orifice will immediately attempt to expand in the confines of the trachea and substantial impaction in this moist environment can readily be envisaged. Presumably, the instillate will act similarly but since it is a fluid it may be propelled forward more effectively into the conducting airways by the simultaneously injected air-stream. Unfortunately, macroscopic dissection does not provide information on the distribution of the dose within the lung lobes and although a substantial difference exists between the powder and solution in terms of the penetration to the lobes it is not possible to differentiate what fraction of the dose reached the respirable regions of the lung relative to the conducting airways. It is of interest to note however, that of the fraction of recovered dose reaching the lung lobes, the distribution within the individual lobes is quite similar for the powder and instillate (Table II). In both cases, deposition was dominant in the right lung compared with the left lung (83.5% vs 16.5%, $p < 0.0045$ powder; 70.1% vs 29.9%, $p < 0.0002$ solution; the mediastinal lobe was considered part of the right lung for this calculation).

Time course studies

rhG-CSF induces changes in the circulating WBC numbers after administration via all of the tested routes of administration (Fig 4). There is also a difference in the response to the two different administered doses (Fig 4). A transient leukopenia is induced that results in an approximately 50% reduction in the WBC counts one hour after dosing. This was especially apparent with the higher dose. In the rat this effect is maximal around 15 min post injection (11). This event also has been observed in patients receiving granulocyte-macrophage colony-stimulating factor (12) and may be associated with an upregulation of the surface expression of adhesion receptors such as CD11b/CD18 (13). Increased pooling and adhesion of the neutrophils within the major organs of the body, particularly the lung, may occur. It has also been noted that stimulated neutrophils exhibit a structural 'stiffening' that renders them less able to pass across the pulmonary capillary bed (14). In effect, the cells are removed from the circulation and will not be sampled during blood collection. By around 3 hrs post dose WBC numbers have returned to normal and thereafter steadily increase to peak at 20–25 hrs after administration of the 500

Table II. The Distribution of Tc-99m Radioactivity Deposited in the Rabbit Lung

Tissue	Powder	Solution
Respiratory tract[a]		
Trachea[b]	49 ± 4**	6.1 ± 1.3
Esophagus	1.3 ± 1.7	0.3 ± 0.4
Mouth	7.3 ± 6.7	0.9 ± 0.8
Lung lobes[c]	28 ± 18**	79 ± 5
Lung lobes[c]		
Upper left	7.4 ± 5.0	4.7 ± 4.4
Lower left	22 ± 12	12 ± 10
Upper right	6.6 ± 4.9	13 ± 10
Middle right	7.0 ± 4.6	20 ± 19
Lower right	51 ± 22	44 ± 7
Mediastinal	5.5 ± 3.8	7.3 ± 2.8
TOTAL Right[d]	70 ± 14**	84 ± 10***
TOTAL Left	30 ± 14	16 ± 10

[a] The percentages of the dose recovered from each part of the respiratory tree. Totals do not add up to 100%. Remaining activity was recovered from residual fluid from dissection and non respiratory components. Error bars are the standard deviation. n = 5.
[b] There was a significant difference in the deposition of powder and solution in both the trachea and lung lobes ($p < 0.01$).
[c] The % distribution of dose that reached the lung lobes.
[d] Total in the right lung includes the percentage deposited in the mediastinal lobe. There was significantly greater deposition in the right vs left lung for both powder ($p < 0.0045$) and solution ($p < 0.0002$).

μg/kg dose and at 15–20 hrs. after the 5 μg/kg dose. The actual dose reaching the circulation is not equivalent in each case, due to differences in the availability of rhG-CSF by the different modes of administration, but generally, the WBC counts peak later and the response is significantly extended with the higher dose: sometimes well beyond 3 days. This extended response was considered unusual as the normal half-life of a neutrophil in the circulation is a matter of hours and most studies have demonstrated a return to baseline in 48–60 hrs after a single dose (2,6). Differentiation of the total WBC counts into the individual cell types reveals that the higher dose of rhG-CSF is also inducing, directly or indirectly, a change in the circulating lymphocyte levels. The lymphocyte counts steadily rise and do not peak until ≈50 hrs post dose (Fig 5). No such effect is observed in animals dosed with 5 μg/kg. The neutrophil levels for both doses are observed to spike within 24 hrs and return to baseline after 2 days (Fig 6). The levels of neutrophils and lymphocytes in the control animals do not change over the time course of the experiments. It is not understood why the lymphocyte population is affected at the higher administered dose and this effect was not observed in hamsters dosed with similar amounts of rhG-CSF (6). Perhaps at this dose level the tissue concentration of rhG-CSF is elevated sufficiently to exert a direct effect on bone marrow progenitor cells that specify lymphocytes as well as granulocytes.

Pharmacokinetic analysis

The plasma concentration vs. time plots of rhG-CSF after IV and SC administration are shown in Fig 7. Plasma concentrations decline rapidly over the first 30 minutes and

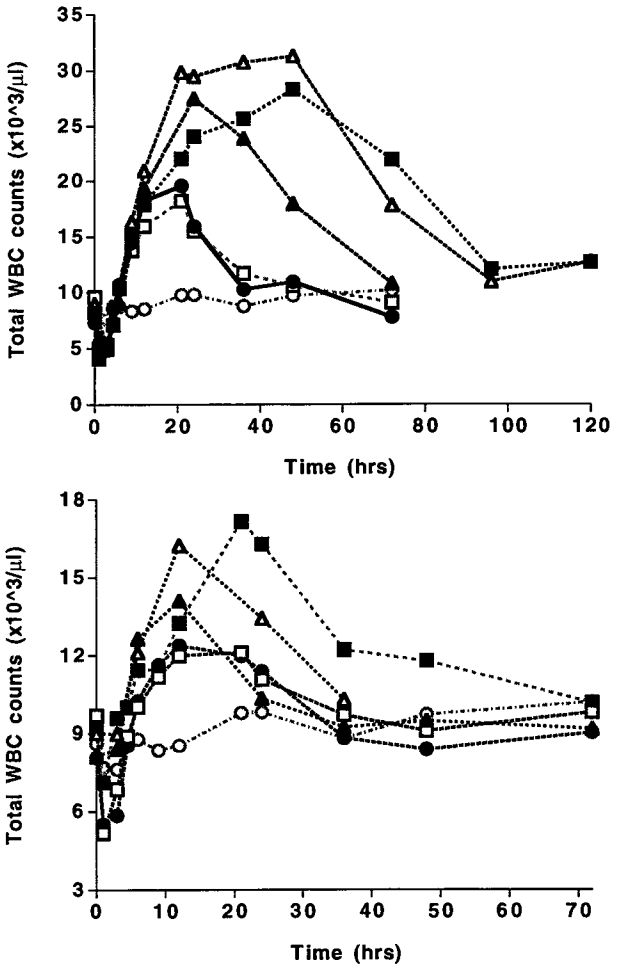


Fig 4 The time course of the WBC response after administration of rhG-CSF by all routes of administration. (A) 500 μg/kg. (B) 5 μg/kg. (■ = SC, □ = IF (G), ▲ = IT, △ = IV, ● = IF (G+), ○ = All controls. n ≥ 5 for each route and dose. Error bars are removed for the purpose of clarity.

this is followed by a prolonged terminal phase. The plots also depict a difference in the terminal slopes for the 2 doses. Further analysis of the IV data shows that the systemic clearance is increased from 11.6 ± 2.6 to 21.8 ± 3.3 ml/hr/kg when the dose is reduced from 500 μg/kg to 5 μg/kg ($p < 0.001$, Table III). This effect cannot be fully explained by any change in the volume of distribution. This suggests that the systemic clearance of the rhG-CSF may include a capacity limited process as has been proposed by Morstyn et al (15). One possible explanation for this process could relate to circulating granulocyte numbers. Studies in rats have shown that clearance increases after repeat doses when neutrophil numbers and activity are elevated (16). Therefore an increase in the number of receptor binding sites on the neutrophil surface and the rate at which endocytosis and degradation occurs may be sufficient to alter the pharmacokinetics of rhG-CSF (17). This may be manifested by an increase in the apparent systemic clearance of the protein as the dose is reduced.

After SC injection a protracted absorptive phase is apparent with T_{max} times of 5.8 hrs after the high dose and 10.5

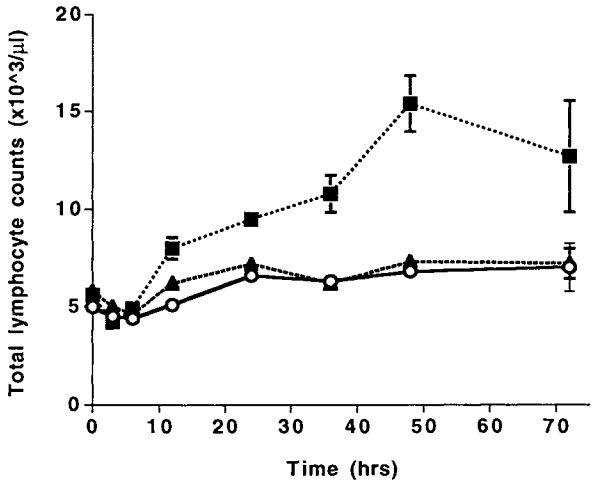


Fig 5 The time course of the overall lymphocyte response after administration of 5 (▲; n = 29) and 500 μg/kg (■; n = 30) rhG-CSF. Values shown are the mean ± sem for all routes of administration at each dose. A steady increase in lymphocyte counts occurs in response to the larger dose that peaks at ≈50 hours and then declines. No response to the lower dose is observed relative to controls (○).

hrs after the low dose (Fig 7, Table IV). After ≈6–8 hrs the plasma values of rhG-CSF are higher than those observed for the respective IV doses. A substantial difference in the apparent bioavailability (F) exists for the two doses (11.0 ± 7.0 vs. 95.3 ± 7.9%). This result differs from observations in rats that the bioavailability of rhG-CSF after SC is ≈75% after 10 and 100 μg/kg doses (18). The variability in total absorption in the rabbit may be associated with the levels of subcutaneous fat. The T_{max} values in rats were observed to be ≈2 hrs compared with the 3–5× longer peak times in the rabbit. Presence of fat may impede the absorption of the protein.

The plasma concentration vs. time profiles after absorption of rhG-CSF from the lung are similar for the instilled

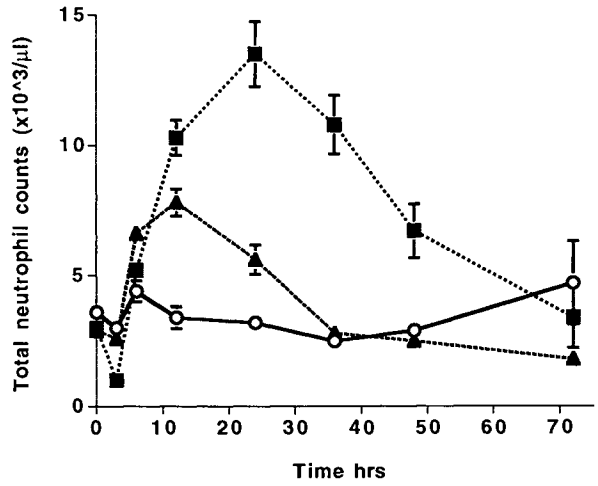


Fig 6 The time course of the overall neutrophil response after administration of 5 (▲; n = 29) and 500 μg/kg (■; n = 30) rhG-CSF. Values shown are the mean ± sem for all routes of administration at each dose. Changes in neutrophil counts is noted at both doses relative to controls (○). The response to the lower dose is abbreviated and the peak is lower and earlier in the time course than for the larger dose.

Table III. IV Pharmacokinetic Parameters[a]

Parameter	Nominal dose (μg/kg) 500	5[b]
$t_{1/2}$ (α) hrs	0.6 ± 0.2	0.3 ± 0.1*
$t_{1/2}$ (β) hrs	4.6 ± 0.2	1.8 ± 0.2***
V_c ml/kg	37 ± 5	47 ± 2**
Cl_s ml/hr/kg	12 ± 3	22 ± 3***
V_{ss} ml/kg	67 ± 11	65 ± 9

[a] Based on 2 compartment iv bolus model.
[b] *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

solution and insufflated powders (Fig 8). Dissolution was apparently not rate-limiting. Peak plasma values are reached in 1 to 2 hrs after dosing. The C_{max} values for the high dose are quite different but this may be a reflection on the dose available for absorption rather than differences in the rate of absorption (Table III). Absorption from the lung is also not the rate-limiting step in the elimination of rhG-CSF from the circulation. This effect is also observed in the hamster (6).

The low pulmonary bioavailability of the powders and solutions is probably due to a low fraction of the administered dose reaching regions of the lung where absorption can take place (Table V). The cascade impactor and lung deposition data demonstrate that a large fraction of the powder will deposit in the upper airways where absorption is likely to be low and mucociliary clearance will be rapid (19). The size of the lung will also play a factor. We previously observed some 60% bioavailability of rhG-CSF in the hamster (6). But the total length of the hamster lung and trachea is less than 3 in. This is in stark contrast to the size of the rabbit lung where the distal tip of the dosing tube inserted into the trachea was still 3 in. from the tracheal bifurcation.

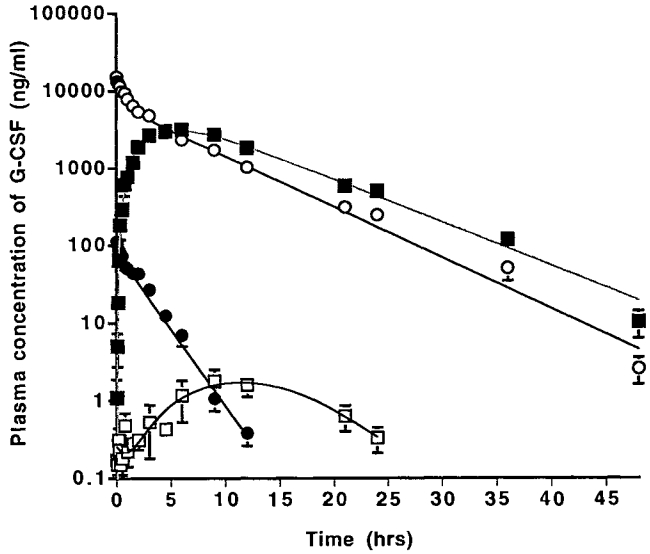


Fig 7 The plasma concentration vs time profiles after administration of 5 and 500 μg/kg rhG-CSF via IV and SC injection. (■ = 500 μg/kg, □ = 5 μg/kg SC; ○ = 500 μg/kg, ● = 5 μg/kg IV). The lines through the IV data represent the best fits generated by nonlinear least squares regression using a biexponential equation as the model. Error bars are the standard error of the mean.

Table IV. Model-Independent Pharmacokinetic Parameters

Treatment group	C_{max}[a] (ng/ml)	T_{max}[a] (hrs)	AUC (μg · hr/ml)	MRT (hrs)
500 μg/kg				
Intravenous inj.	—	—	48 ± 8	5.9 ± 0.8
Subcutaneous inj.	3193 ± 385	5.8 ± 2.0	46 ± 6	11 ± 1
Solution instillation	375 ± 31	1.1 ± 0.3	2.6 ± 0.9	5.4 ± 0.5
G insufflation	66 ± 16	1.5 ± 0.6	0.4 ± 0.2	4.4 ± 0.8
G+ insufflation	42 ± 7	1.2 ± 1.1	0.3 ± 0.1	4.2 ± 1.0
5 μg/kg			$\times 10^{-3}$	
Intravenous inj.	—	—	206 ± 93	2.8 ± 0.7
Subcutaneous inj.	1.8 ± 0.7	10.5 ± 1.6	24 ± 15	12 ± 2
Solution instillation	5.9 ± 0.7	1.7 ± 0.4	26 ± 10	3.1 ± 0.2
G powder insufflation[b]	2.7 ± 1.2	1.0 ± 0.5	18 ± 9	4.0 ± 0.5
G+ powder insufflation[b]	3.1 ± 0.4	1.5 ± 0.3	11 ± 4	2.8 ± 0.5

[a] C_{max} and T_{max} data were taken from the observed data.
[b] G = rhG-CSF powder, G+ = rhG-CSF powder + excipients.

It is also feasible that some aggregation of rhG-CSF could take place in the lung lining fluids over time. The protein is known to aggregate over time in an isotonic salt environment and will eventually produce insoluble aggregates. This effect is also concentration dependent (unpublished data). Aggregation may occur in the upper airway where powder agglomerates have deposited and the local concentration of the dissolving protein is high. However, powder will have been well dispersed and deaggregated if it has penetrated the alveolar regions where the presence of natural surfactant may aid dissolution and inhibit aggregation.

Since, it is evident that the clearance of rhG-CSF from the circulation is dose-dependent, the estimates of bioavailability also must be interpreted with caution. The terminal slopes of all concentration vs. time profiles excepting the high IV and SC doses are similar. Hence, F values were obtained relative to the IV 25 μg/kg dose. The F value for the 500 μg/kg SC dose was obtained using the 500 μg/kg IV data.

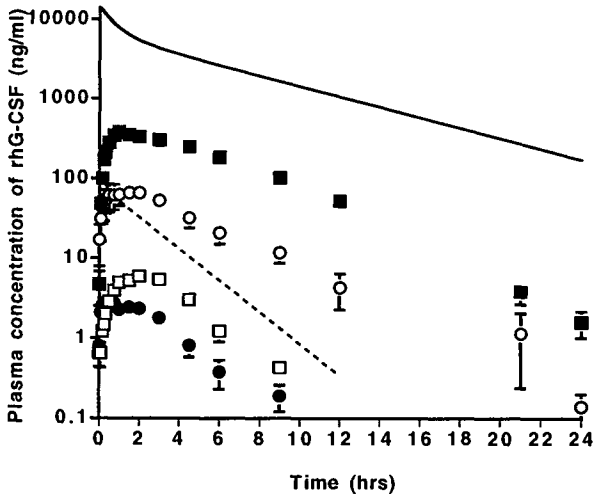


Fig 8 The plasma concentration vs time profiles after administration of 5 and 500 μg/kg rhG-CSF via instillation and insufflation (■ = 500 μg/kg, □ = 5 μg/kg instillation; ○ = 500 μg/kg, ● = 5 μg/kg G insufflation). The solid line and dashed line represent the best fits for the high and low dose IV data respectively. Error bars are the standard error of the mean.

A better gauge of the absorptive capacity of the protein may be obtained after aerosol administration of powder and solution. Aerosol should deposit homogeneously over the lung surface (20) that, in effect, is mainly alveolar surface. For example, Colthorpe et al. (21) have noted an approximately 10× increase in the bioavailability of insulin after aerosol administration compared to intratracheal instillation. They also raise the point that mucociliary clearance may affect the absorption of instillate to a greater degree than the aerosol. If drug is significantly absorbed from the conducting airways over time then this parallel clearance mechanism may influence the concentration vs. time profiles observed in the circulation. If negligible absorption takes place in the airways then the impact of mucociliary clearance will be minimal. In both scenarios however, it will not be possible to get an accurate idea of the available dose. In addition, movement of instilled fluid within the respiratory tree in association with breathing and body movement may transport dose to and from sites from where it can absorb. Thus, instillation and insufflation, although simple to perform, may not be the ideal approach for assessment of bioavailability. The techniques might be better suited for an initial assessment of absorption and/or to indicate whether a local or systemic response is effected. Ideally, for experimental studies some method is needed that will deliver a quantitative bolus aerosol that is well distributed over the lung surface.

To conclude, we have shown that rhG-CSF can be prepared in powder formulations suitable for inhalation purposes and that they retain *in vitro* and *in vivo* bioactivity. Powders insufflated to the lungs of rabbits using a simple technique were shown to induce a pharmacological response. Absorption of the powders was not dissolution rate-limited and apparently more rapid than absorption from a subcutaneous dose. The results suggest that it may be possible to administer rhG-CSF to patients as a dry powder formulated product. Clearance of rhG-CSF from the circulation may be saturable but a range of doses need to be studied to identify what mechanisms are responsible.

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Table V. Apparent Bioavailability (F and F_{lung}) of rhG-CSF[a,b]

	Nominal dose (μg/kg)			
	500[c]		5	
Parameter	F	F_{lung}	F	F_{lung}
Subcutaneous inj.	95.3 ± 7.9	—	11.0 ± 7.0	—
Solution instillation	6.2 ± 2.2	10.3 ± 3.9	10.6 ± 4.0	17.7 ± 7.1
G insufflation	0.7 ± 0.7	4.9 ± 6.0	4.8 ± 1.9	32.9 ± 16.4
G+ insufflation	2.7 ± 1.5	18.3 ± 12.5	2.0 ± 1.1	13.6 ± 9.3

[a] F is the bioavailability based on the administered dose. F_{lung} is the bioavailability based upon the dose reaching the lung lobes. For solution instillation this was estimated as 60.1% of the dose. For powder insufflation this was estimated as 14.7% of the dose.

[b] Since there is a possibility that the clearance kinetics of rhG-CSF are nonlinear with dose, the use of bioavailability as calculated must be treated with caution. For these calculations the AUC for the 500 μg/kg dose SC dose was compared directly with the AUC for the equivalent IV dose. All remaining calculations were based upon the AUC for the 5 μg/kg IV dose.

[c] The high dose for the G+ powder was 75 μg/kg. This allowed the total mass of administered powder to remain at 1.5 mg.

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