Characterization of Aerosols of Human Recombinant Deoxyribonuclease I (rhDNase) Generated by Jet Nebulizers

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Recombinant human deoxyribonuclease I (rhDNase) is a new therapeutic agent developed to improve clearance of purulent sputum from the human airways. It is delivered by inhalation. Four jet nebulizers, T Up-Draft II (Hudson), Customized Respirgard II (Marquest), Acorn II (Marquest), and Airlife Misty (Baxter), were evaluated *in vitro* for their ability to deliver aerosols of rhDNase. The aerosols were generated from 2.5-mL aqueous solutions of rhDNase, at concentrations of either 1 or 4 mg/mL. In all experiments, the Pulmo-Aide Compressor (De Vilbiss) was used to supply the air to the nebulizers. Between 20 and 28% of the rhDNase dose initially placed in the nebulizers was delivered to the mouthpiece in the respirable range (1–6 μm). Evaluation of the rhDNase following nebulization in all four devices indicated that there was no loss in enzymatic activity and no increase in aggregation. Circular dichroism spectrophotometry indicated there was no change in either the secondary or the tertiary structure in rhDNase following nebulization. These results show that all four nebulizers are essentially equivalent in their ability to deliver respirable doses of rhDNase in an intact, fully active form. Changing the concentration of the solution in the nebulizer from 4 to 1 mg/mL rhDNase leads to a proportional reduction in the respirable dose delivered to the mouthpiece.

KEY WORDS: inhalation therapy; rhDNase; aerosols; jet nebulizers; therapeutic proteins.

INTRODUCTION

Recombinant human pancreatic deoxyribonuclease I (rhDNase) is a novel respiratory drug. It was found to improve *in vitro* the rheological properties of purulent sputum from cystic fibrosis (CF) patients (1). The reduction of viscosity was associated with a decrease in size of DNA derived from human neutrophils which infiltrate the airways in response to bacterial infections. Short-term inhalation of aqueous solutions of rhDNase by CF patients was well tolerated (2,3). The therapy was associated with a significant improvement in pulmonary function tests (2,3). The generation and the subsequent delivery of the rhDNase aerosol to the airways are critical to the ability of rhDNase to decrease the viscoelasticity of lung secretions (1). The aerosol size distribution is an important variable in defining the site of droplet or particle deposition in the patient (4). A distribution of particles between 1 and 6 μm in size should give a relatively uniform deposition of rhDNase in the airways (5–7). Droplets larger than 6 μm will deposit mainly in the oropharynx, whereas droplets less than 1 μm are likely to be exhaled during normal tidal breathing (3,4). Although the choice is somewhat arbitrary, this portion (1–6 μm) of the size spectrum is referred to as the "respirable fraction" (RF).

Jet nebulizers cannot effectively aerosolize all of the rhDNase solution because some of it remains in the "dead volume" of the equipment. The fraction of the dose initially placed in the nebulizer that is actually aerosolized and delivered to the mouthpiece during the nebulization time (10 min) is termed the "nebulizer efficiency" (E). A more accurate indication of the overall efficiency of delivering the aerosol to the target airways is then the product of the respirable fraction times the nebulizer efficiency, or the "delivery" (DE):

$$DE = E \times RF$$

This parameter estimates the potential of delivery of the aerosol to the target airways. The actual delivery will vary depending on the severity of the disease as well as the pulmonary function of the individual patient.

To assure consistent delivery of rhDNase, the various nebulizers tested should have similar values for the measured delivery. Moreover, most proteins are surface active agents, and the effect of protein concentration on droplet size distribution and nebulizer efficiency needs to be addressed.

Jet nebulizers generate an aerosol from a liquid by introducing a fast stream of air through the solution. Over 97% of the primary aerosol generated is of large nonrespirable droplets that impact on the surfaces and baffle system of the nebulizer and are recirculated (4). Proteins are generally surface active molecules and are susceptible to surface denaturation at air water interfaces (8). Denaturation of a protein may also expose hydrophobic amino acid residues normally found in the interior of the protein. The exposure of hydrophobic residues can lead to aggregation of the protein, which may result in reduced activity. An additional possibility is the generation of potentially immunogenic species by virtue of either unfolding and/or aggregation of the protein. This of course is a major concern with proteins that are to be administered chronically such as rhDNase. Denaturation of a globular protein may also affect water structure, which in turn might alter surface tension properties of the rhDNase solution. Alteration of surface tension could affect the droplet size distribution, which would alter the amount of rhDNase that reaches the airways. Thus, it is important to determine the effect of the nebulization process on rhDNase.

The objectives of this work were to determine the size distribution and overall efficiency of delivery of rhDNase aerosols generated by four different jet nebulizers. In addition, we assessed the effects of the nebulization process on the physical and biochemical integrity of rhDNase.

MATERIALS AND METHODS

The following disposable nebulizers were tested: T Up-Draft II Model 1734 (Hudson RCI, Temecula, CA), Airlife Misty with Tee Adapter Model 002038 (Baxter-American Pharmaseal Company, Valencia, CA), Customized Respirgard II Model 124030 modified by removal of the blue expi-

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ratory one-way valve (Marquest, Englewood, CO), and Acorn II Model 124014 (Marquest, Englewood, CA).

Generation of the Aerosols

rhDNase solutions at ~pH 7.0 were supplied as 2-mL vials of 4 mg/mL (0.125 m*M*) rhDNase formulated in 8.77 mg/mL (150 m*M*) NaCl and 0.11 mg/mL (1 m*M*) $CaCl_2$. The formulation vehicle was used to dilute this solution to 1 mg/mL where appropriate. The experimental setup for generation of the aerosols was designed to duplicate the conditions used in the clinical trials as closely as possible. In each experiment, 2.5 mL of rhDNase solution was placed in the nebulizer cup. The compressor (Pulmo-Aide Model 5610D, DeVilbiss, Somerset, PA), which generated a nominal airflow rate of 7 L/min at a nominal pressure of 43 psi, was turned on and the nebulization was allowed to proceed for 10 min. In all experiments, the nebulization was complete within this period of time as observed by cessation of aerosol generation.

Experimental Setup for Droplet Size Analysis

Prior to setup, the individual stages of a seven-stage cascade impactor (In-Tox Products, Albuquerque, NM) were cleaned, glass plates were placed on each brass disk, and the stages and the filter (Type A/E, Gelman, Ann Arbor, MI) were weighed on an electronic analytical balance with a resolution of 0.1 mg and a repeatability to 0.1 mg (S/P 120, American Scientifics Products, McGaw Park, IL). The cascade impactor was then assembled and placed in a laminar flow hood to ensure that the air drawn through the cascade impactor was free of interfering particulates. In a control experiment, room air alone was passed through a dry cascade impactor for 24 hr, and the amount of material collected in the impactor was found to be insignificant. A vacuum source was used to draw the aerosol through the cascade impactor at 8.0 L/min, as monitored by an in-line mass flow meter (Model 821, Sierra Instruments, Monterey, CA). A 0.2-μm nylon filter (MSI Calyx, Westboro, MA) was placed in-line to protect the flow meter from any liquid residue. The higher flow rate through the impactor (8 L/min) ensured that all the rhDNase aerosol (generated at 7 L/min) was continuously drawn through the cascade impactor. The nebulizer was connected to the compressor with the tubing provided by the nebulizer manufacturer. The aerosol generated in the nebulizer was directed to the cascade impactor through a short, flexible 6-in. piece of tubing.

Calculation of Aerosol Parameters

The cascade impactor used in these studies has effective cutoff diameters (ECD) from 8 to 0.5 μm at 5 L/min flow. However, in our work it was used exclusively at 8.0 L/min. The ECD for each stage at 8.0 L/min was calculated by multiplying the manufacturer specified ECD's at 5 L/min by $(5/8)^{1/2}$ based on the theory that the ECD is inversely proportional to the square root of the flow rate (9). This gave the new ECDs as 6.11, 3.84, 2.42, 1.51, 0.96, 0.60, and 0.37 μm for stages 1–7, respectively. As the main aspect of the studies reported here was to inquire about the *in vitro* equivalence of the nebulizers tested, the absolute accuracy of the sizing procedure was not essential.

Determination of Mass Distribution. Following the 10-min nebulization, the nebulizer was disconnected and 8.0 L/min of filtered air was allowed to pass through the cascade impactor overnight (between 18 and 22 hr). Pilot experiments showed that complete drying of the individual stages and the filter occurred within this time frame. The cascade impactor was then disassembled, and the stages and the filter were reweighed on an analytical balance. The dry weight of the excipients and rhDNase on each stage and on the filter was determined by subtracting the initial weight from the final weight. In addition, the rhDNase solution remaining in the nebulizer and on the walls of the tubing was transferred to a plastic weigh boat and allowed to dry in a desiccant-filled jar. This weigh boat was reweighed, allowing the dry weight of the undelivered excipients and rhDNase to be determined. The sum of the dry weights of both the delivered and the undelivered materials provides a means of ensuring mass balance; that is, the dry weight after the experiment should equal the dry weight of the excipients and rhDNase in the initial 2.5-mL solution. This was determined experimentally by drying 2.5 mL of the 1 and 4 mg/mL rhDNase formulations.

An assumption inherent in our analysis is that rhDNase and excipients distribute equally in all aerosol particles and, thus, that the percentage dry weight of either species on each stage and the filter is identical to the percentage of combined weight on that location. The proportion of rhDNase to excipients in an aerosol particle should be independent of the particle size and dependent only upon the concentration in the reservoir solution. This assumption should be valid as long as neither the drug nor the excipients are lost in the nebulizer (due to absorption, adsorption, precipitation, evaporation, etc.). The experimental verification of the constancy of the proportion of rhDNase to the excipients was carried out by assaying sodium ions and rhDNase before and after nebulization.

Nebulizer Efficiency, E. E was determined by dividing the accumulated dry weight on the cascade impactor stages and the filter by the total dry weight originating from the initial 2.5-mL rhDNase solution and multiplying by 100.

Respirable Fraction, RF. RF was calculated by summing the accumulated dry weight on stages 2 through 5 (6.11 to 0.96 μm), dividing this by the accumulated dry weight on all the stages and the filter, and multiplying by 100.

Statistical Analysis. The total delivery of respirable droplets to the mouthpiece DE from four jet nebulizers was subjected to analysis of variance (10). The Kruskal–Wallis test was used to determine if nebulizers had equivalent performance (the null hypothesis of nebulizer equivalence was rejected for $P \leq 0.003$).

Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (σ_g). MMAD and σ_g are often used to characterize the "average" particle diameter and the "width" of the particle size distribution, respectively (4,9). The latter is meaningful for aerosols in which the size distribution conforms to logarithmic-normal distribution function. Following the procedure outlined above to determine the mass on each stage, the cumulative mass below ECD was plotted on a log-probability scale (9). The MMAD

and σ_g were obtained from the linear regression line of the cumulative mass below ECD on a probability scale vs the logarithm of the aerodynamic diameter. The MMAD was the diameter corresponding to the 50% point in the cumulative distribution, and σ_g was calculated as MMAD divided by the diameter corresponding to 84.1% of the cumulative percentage (9).

Determination of the Properties of the rhDNase in the Aerosol

The same procedure for generation and collection of aerosols was followed as reported in the previous section, with one major difference. In place of the cascade impactor was an arrangement designed to collect the aerosol particles by impaction in a test tube. This test tube was placed in an ice-filled 250-mL sidearm flask to minimize potential degradation of rhDNase. The aerosol was delivered to the test tube via a narrow flexible piece of tygon tubing (3/16-in. id) attached to a 2.0-mL plastic pipette. The tip of the pipette was inserted into the test tube just above the bottom. The collection efficiency, by impaction, was maximized by using a pipette with a narrow-diameter tip and a relatively high aerosol flow rate. The rhDNase solution collected in the test tube, as well as that remaining in the nebulizer reservoir, was then analyzed as described in the next section.

Assays

UV Spectroscopy. The rhDNase solution was diluted to about 0.5 mg/mL, loaded into a 1-cm quartz cuvette, and the absorbance read in a diode array spectrophotometer (Model 8451, Hewlett Packard, Mountain View, CA). The concentration of rhDNase was then determined using an absorptivity of 1.7 cm^{-1} $(mg/mL)^{-1}$ at 280 nm without correcting for light scattering. Absorbance due to light scattering at 320 to 400 nm, indicative of high molecular weight aggregates, was also measured. The Rayleigh light-scattering correction method was applied to samples showing high scattering to determine the corrected rhDNase concentration (11).

pH. The pH of the rhDNase solutions was measured with a microelectrode (Microelectrodes, Inc., Londonderry, NH) using a pH meter (Model pHM84, Radiometer, Westlake, Ohio).

rhDNase Methyl Green Assay. This is a microtiter plate assay version of a method adapted from Kurnick (12). rhDNase samples were diluted sequentially into assay diluent [25 m*M* HEPES, 4 m*M* $CaCl_2$, 4 m*M* $MgCl_2$, 0.1% bovine serum albumin (BSA), 0.01% Thimerosol, 0.05% Polysorbate 20, pH 7.55 ± 0.05] to between 0.2 and 0.8 μg/mL. The active concentrations at each dilution were multiplied by appropriate dilution factors and averaged. The averaged values from at least three determinations were normalized to account for assay variation by dividing by the value for a reference sample that was submitted for assay at the same time.

Sizing Chromatography. The amounts of rhDNase monomer and aggregated rhDNase were determined on a 7.8 mm × 30-cm TSK 2000SWXL silica-based column (Hewlett Packard, Mountain View, CA). The flow was isocratic at 1.0 mL/min of mobile phase (5 m*M* HEPES, 150 m*M* NaCl, 1 m*M* $CaCl_2$, titrated to pH 7.0) for 15 min. Absorbance was monitored at 214 nm. Peak areas and retention times were recorded. The elution times of the gel filtration standards (Bio-Rad, Richmond, CA), consisting of thyroglobulin (670 kD), γ-globulin (158 kD), ovalbumin (44 kD), myoglobin (17 kD), and cyanocobalamin (1.35 kD), were used to distinguish between the monomer rhDNase and any aggregated species. The values for percentage monomer were determined from the peak areas and are reported as the mean result of duplicate injections.

Sodium Ion Concentration. The concentration of sodium in the rhDNase solutions was determined by flame photometry (Model IL943, Instrumentation Laboratories, Lexington, MA).

Circular Dichroism Spectrophotometry. These tests were carried out on the aerosols generated from 1 mg/mL rhDNase solutions. An Aviv (Lakewood, NJ) circular dichroism spectrometer, Model 62DS, with an attached Lauda RCS (Brinkmann Instruments, Inc., Burlingame, CA) water bath providing sample cell temperature control was used to obtain both near-UV (240 to 360 nm) and far-UV (196 to 250 nm) CD spectra of rhDNase samples. All nebulized and control rhDNase samples were diluted with formulation buffer to between 0.25 and 0.4 mg/mL rhDNase. The concentration was determined by UV spectroscopy just prior to circular dichroism analysis. Samples were placed in a 1 or a 0.01 cm pathlength quartz cell for the near- and far-UV CD analysis, respectively. The sample cell was held constant at 20°C throughout the analysis. The CD spectra were collected every 0.2 nm, with a 5 sec averaging time at a 1.5 nm bandwidth. Triplicate scans were averaged for each sample. The individual repeat scans were compared to ensure that the sample was not perturbed during the course of analysis. The results were converted to mean residue weight ellipticity, Θ, using a mean residue weight of 112.5 for rhDNase.

RESULTS AND DISCUSSION

Nebulizer Characterization and Performance

More than 95% of the aerosol generated by the nebulizer reached the cascade impactor. This indicates that the tubing did not significantly alter the aerosol flow profile or particle size distribution. The aerosol reaching the cascade impactor was thus representative of the aerosol that would reach the patient's mouth.

The total weight of dry components (drug plus involatile excipients) in 2.5 mL of the 1 mg/mL rhDNase formulation was calculated theoretically to be 24.7 mg and was experimentally verified as 25.0 ± 0.6 mg (n = 4); for the 4 mg/mL rhDNase, the theoretical weight was 32.2 mg, which agreed within the experimental accuracy with the total mass collected of 34.2 mg.

The total recovery of dry rhDNase plus excipients at the end of each cascade impaction run (i.e., the sum of dry masses of deposits from the cascade impactor, the nebulizer and the connecting tubing) was on average >89% for all nebulizers at both protein concentrations. Thus, the majority of the rhDNase used in these experiments could be accounted for by mass balance considerations, indicating that the size analysis is representative of the aerosol that would be delivered to the patients.

The aerosol particle size distribution characterized by cascade impactor studies revealed minor differences between the aerosols generated by the four devices at either 1 or 4 mg/mL; the concentration of rhDNase had no apparent effect on the particle size distributions (Figs. 1a and 1b). In all cases the respirable fraction (i.e., the fraction of dry weight accumulated on stages 2–5, approximately 1–6 μm) was 46–53% for each nebulizer (Table I). It can be noted that expressing the results in terms of an experimentally defined "respirable fraction" avoids the need to postulate a particular shape of the size distribution function (such as logarithmic-normal).

The plots of cumulative mass, on probit scale, against the logarithm of aerodynamic size were approximately linear. Frequently these plots deviated from linearity in the region corresponding to the smallest droplet size range, and these data points were omitted from the regression analysis. The amount of material deposited on the stages in this size range was less than 10% of the total mass, and therefore the accuracy and precision of these low masses were relatively poor. Also, it is likely that some evaporation of these small droplets was taking place at these most distant plates of the impactor since these droplets appeared to be below the sizes extrapolated from the data at higher sizes. Inclusion of these does not affect significantly the determination of the MMAD. All four devices generated aerosols described by an MMAD of 3.9–4.9 μm; approximate values of σ_g ranged from 3.4 to 3.9, indicating significant heterodispersity (5–7) (Table II). The relatively high values of σ_g are probably the result of evaporation of the smaller droplets (13). This phenomenon has little effect on the respirable fraction (unpublished results), which, as pointed out above, is a more robust description of droplet size distribution.

The four types of devices delivered ~50% of the initial loading of rhDNase and excipients to the cascade impactor (nebulizer efficiency; Table I). The incompleteness of delivery is well recognized for drug solutions aerosolized by jet nebulizers (4,14–16). The reason for this phenomenon is twofold. First, the volume of rhDNase solution remaining in the reservoir following nebulization is typically 0.6 to 0.9 mL of the original 2.5-mL sample load (Table III). Second, the rhDNase solution evaporates during nebulization because of contact with the dry air stream (Tables III and IV), so that more drug remains in the reservoir than would be expected from the "dead volume." In fact, rhDNase and sodium concentration determinations verify that both components concentrate proportionately during nebulization (as shown by the sodium-to-rhDNase molar ratio; Table IV).

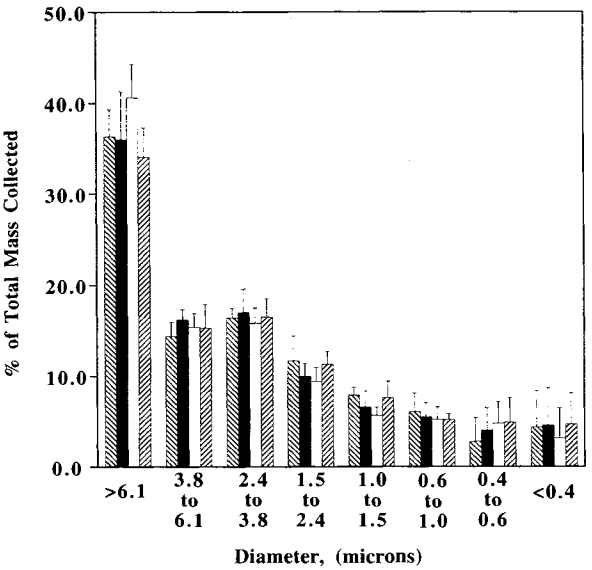

Fig. 1a. Particle size distribution of nebulized rhDNase (1 mg/mL). (▨) Respirgard II, n = 8; (■) Acorn II, n = 8; (□) Baxter, n = 7; (▨) Hudson, n = 8.

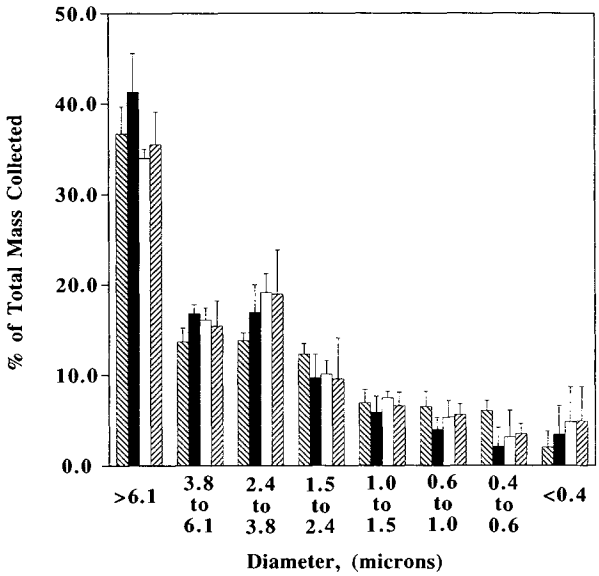

Fig. 1b. Particle size distribution of nebulized rhDNase (4 mg/mL). (▨) Respirgard II, n = 7; (■) Acorn II, n = 8; (□) Baxter, n = 5; (▨) Hudson, n = 6.

Table I. 1 and 4 mg/mL rhDNase Delivery by Jet Nebulizers

Nebulizer	n^a	% respirable fraction	% nebulizer efficiency	% delivery
1 mg/mL				
Marquest Customized Respirgard II	8	51 ± 3	48 ± 6	24 ± 4
Marquest Acorn II	8	50 ± 4	55 ± 6	27 ± 3
Hudson T Up-Draft II	8	51 ± 4	49 ± 5	25 ± 2
Baxter Airlife Misty	7	46 ± 3	44 ± 2	21 ± 1
4 mg/mL				
Marquest Customized Respirgard II	7	47 ± 2	46 ± 7	22 ± 4
Marquest Acorn II	8	49 ± 4	53 ± 9	26 ± 4
Hudson T Up-Draft II	6	51 ± 4	52 ± 5	27 ± 4
Baxter Airlife Misty	5	53 ± 2	44 ± 3	23 ± 2

[a] Values are the result of n independent measurements using n individual nebulizers, given as the mean value and standard deviation.

Table II. Characterization of 1 and 4 mg/mL rhDNase Aerosols Generated by Jet Nebulizers[a]

Nebulizer	n^b	MMAD (μm)	σ_g
1 mg/mL			
Marquest Customized Respirgard II	8	4.1 ± 0.5	3.4 ± 0.5
Marquest Acorn II	8	4.0 ± 0.4	3.5 ± 0.3
Hudson T Up-Draft II	8	3.9 ± 0.3	3.5 ± 0.5
Baxter Airlife Misty	7	4.7 ± 0.6	3.5 ± 0.4
4 mg/mL			
Marquest Customized Respirgard II	7	3.9 ± 0.5	3.9 ± 0.3
Marquest Acorn II	8	4.9 ± 0.6	3.1 ± 0.2
Hudson T Up-Draft II	6	4.0 ± 0.5	3.3 ± 0.6
Baxter Airlife Misty	5	3.9 ± 0.2	3.3 ± 0.3

[a] Values given are the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (σ_g).

[b] Values are the result of n independent measurements using n individual nebulizers, given as the mean value and standard deviation.

The total delivery of respirable droplets to the mouthpiece DE from the four nebulizers ranged on average between 22 and 27% for the 4 mg/mL formulation (Table I); the small differences between the nebulizers were not statistically significant. The interbrand variation was similarly narrow when the 1 mg/mL formulation was tested, ranging from 21 to 27% for the four devices (Table I). The Hudson and the two Marquest jet nebulizers were essentially equivalent at delivering 1 mg/mL rhDNase. When these results were subjected to analysis of variance (10), the Baxter jet nebulizer appeared to be slightly less efficient than the other three nebulizers at delivering 1 mg/mL rhDNase. Decreasing the concentration of rhDNase from 4 mg/mL to 1 mg/mL in the nebulizer solution had no effect on the overall percentage delivered by the four devices (Table I); therefore, the reduction in concentration from 4 to 1 mg/mL led to a proportional (fourfold) reduction in the respirable dose delivered to the mouthpiece.

Table III. Recovery of rhDNase (Initial 1 mg/mL) in Protein Characterization Experiments

rhDNase sample	rhDNase concentration (mg/mL)	Volume (mL)
Before nebulization	1.06	2.5
Marquest Acorn II		
Residua after nebulization	1.45	0.613
Collected aerosol	1.22[a]	0.646
Marquest Customized Respirgard II		
Residua after nebulization	1.35	0.686
Collected aerosol	1.20	0.930
Hudson T Up-Draft II		
Residua after nebulization	1.28	0.721
Collected aerosol	1.11	0.888
Baxter Airlife Misty		
Residua after nebulization	1.38	0.854
Collected aerosol	1.05	0.880

[a] This sample was corrected for light scattering (≈10%).

Properties of the rhDNase in the Aerosol and in the Residua in the Nebulizer

The collection of the aerosol in the experiments for protein and sodium assays was incomplete (Table III), mainly because some of the aerosol escaped capture by impaction in the test tube. However, subsequent experiments with modified aerosol capture equipment (17) showed that the additional material recovered did not differ in its composition from the rest of the aerosol.

The enzyme fully retained its activity during the nebulization process as evidenced by the methyl green assay of the collected material, as well as in the residue in the nebulizer after cessation of the aerosol generation (Table V). The active concentration (determined by methyl green activity assay) of rhDNase when divided by its concentration in solution (as determined by UV spectroscopy) was the same in the collected nebulized samples and in the reservoir residua (Table V). The increase in the rhDNase concentrations in the reservoir solutions (Table IV) was proportionately matched by an increase in the active concentrations. Evaporative losses from jet nebulizers have been well documented in the past (4,13–16).

As mentioned previously, aggregation could lead either to a decrease in protein activity or to an immunogenic protein. One of the few thorough studies investigating the effect of nebulization on a protein was done with human growth hormone (hGH) (18). Formulations of rhGH without surfactant were highly aggregated after nebulization. It was also noted that the protein remaining in the nebulizer bowl was even more highly aggregated than the collected aerosol. This result is not surprising since the residual protein has been recirculated and subjected to continuous nebulization throughout the delivery period. Inclusion of a surfactant in the formulation greatly decreased the amount of particulates and soluble aggregate that formed as a result of the nebulization process. Significantly, even without inclusion of a surfactant in the rhDNase formulation, SDS-PAGE and sizing chromatography revealed that essentially 100% of the rhDNase eluted as monomer after nebulization.

For the 1 mg/mL formulation, additional tests of the protein properties were carried out. Near-UV circular dichroism spectrophotometry provides information on the environment of specific aromatic amino acids present in a protein, i.e., the tertiary structure of the protein. Differences between spectra (as observed by peak or shoulder shifts) for a given protein generally indicate that one or more of the aromatic residues change their accessibility to the polar solvent or are constrained to a different extent by surrounding residues (19). The near-UV circular dichroism spectrum of rhDNase was unaffected by nebulization in the Acorn II (Marquest), T Up-Draft II (Hudson), and Airlife Misty (Baxter) jet nebulizers (a representative CD spectrum is shown in Fig. 2a). This suggests that the overall tertiary structure of rhDNase is unaffected by nebulization in these devices. Optical activity in the far-UV region of a circular dichroism spectrum is dominated by the peptide backbone, i.e., the secondary structure of the protein (19). Nebulization in the Acorn II (Marquest), T Up-Draft II (Hudson), and Airlife Misty (Baxter) jet nebulizers resulted in no change within experimental error in the far-UV circular dichroism spec-

Table IV. rhDNase (1 and 4 mg/mL Initial) and Sodium Concentrations Before and After Jet Nebulization

rhDNase sample	1 mg/mL			4 mg/mL		
	rhDNase concentration (mg/mL)[a]	Na (m*M*)[b]	Na/rhDNase (mole ratio)[c]	rhDNase concentration (mg/mL)[a]	Na (m*M*)[b]	Na/rhDNase (mole ratio)[c]
Before nebulization	1.06	156 ± 5	4300	3.75	127.1 ± 1.0	990
Marquest Acorn II						
Residua after nebulization	1.45	208 ± 8	4200	ND	ND	ND
Collected aerosol	1.22[d]	162 ± 4	3900	ND	ND	ND
Marquest Customized Respirgard II						
Residua after nebulization	1.35	193 ± 4	4200	ND	ND	ND
Collected aerosol	1.20	163 ± 6	4000	ND	ND	ND
Hudson T Up-Draft II						
Residua after nebulization	1.28	184 ± 2	4200	4.25	150.3 ± 0.4	1040
Collected aerosol	1.11	151 ± 3	4000	4.01	141 ± 2	1030
Baxter Airlife Misty						
Residua after nebulization	1.38	195 ± 3	4200	4.38	151 ± 2	1010
Collected aerosol	1.05	145 ± 3	4000	3.96	138 ± 2	1020

[a] Concentration determined by ultraviolet absorption spectroscopy using an absorptivity of 1.7 $(mg/mL)^{-1}\ cm^{-1}$, which was determined by quantitative amino acid composition analysis.
[b] Sodium concentrations are the mean ± SD of triplicate samples.
[c] Weight concentrations of rhDNase were converted to moles of rhDNase using a protein molecular weight of 29,339 based on the expected molecular weight from protein sequence as coded by cDNA sequence.
[d] This sample was corrected for light scattering (≈10%).

trum of rhDNase (an example is shown in Fig. 2b). This suggests that the secondary structure of rhDNase was unperturbed by nebulization in these devices. The samples nebulized in the Customized Respirgard II (Marquest) were not analyzed and are assumed to yield spectra similar to those for the Acorn II nebulized samples, as the nebulizer bowl and baffling system are identical to those of the Acorn II device.

Changes in the pH as a result of the nebulization procedure could indicate chemical degradation which was not detected by other techniques. An average decrease of 0.5 pH unit was observed for the collected nebulized samples compared to that of the reservoir residua for the 1 mg/mL formulation. The pH drop disappeared in experiments performed exclusively in a nitrogen environment, suggesting that absorption of CO_2 from the air by the fine aerosol mist (resulting in the formation of carbonic acid) caused the drop in the pH of the solutions. Since the protein itself acts as a buffer, only a very small pH decrease was observed in the studies at the higher concentration of 4 mg/mL rhDNase solutions. Conversely, a larger decrease was observed for studies with the formulation vehicle alone, providing further

Table V. rhDNase Formulation (1 and 4 mg/mL) Before and After Delivery by Jet Nebulization

rhDNase sample	1 mg/mL		4 mg/mL	
	Activity[a]	% monomer	Activity[a]	% monomer
Before nebulization	1.00 ± 0.10	100 ± 0.1	1.00 ± 0.10	99.9 ± 0.1
Marquest Acorn II				
Residua after nebulization	0.94 ± 0.10	100 ± 0.1	ND	ND
Collected aerosol	0.93 ± 0.10	98.5 ± 0.7	ND	ND
Marquest Customized Respirgard II				
Residua after nebulization	1.01 ± 0.10	100 ± 0.1	ND	ND
Collected aerosol	0.96 ± 0.10	100 ± 0.1	ND	ND
Hudson T Up-Draft II				
Residua after nebulization	1.00 ± 0.10	100 ± 0.1	1.01 ± 0.10	99.9 ± 0.1
Collected aerosol	0.90 ± 0.10	100 ± 0.1	1.00 ± 0.10	99.6 ± 0.1
Baxter Airlife Misty				
Residua after nebulization	1.01 ± 0.10	100 ± 0.1	0.97 ± 0.10	99.9 ± 0.1
Collected aerosol	0.96 ± 0.10	100 ± 0.1	0.96 ± 0.10	99.9 ± 0.1

[a] Normalized activity expressed as ratio of active concentration of rhDNase as determined by methyl green activity assay to concentration determined by ultraviolet absorption spectroscopy relative to a value of 1.00 for the control rhDNase sample. The error in the value for the normalized activity is about 10%. The active concentration is an average of six replicates assayed in the methyl green assay. The concentration determined by UV spectroscopy is based on one measurement only.

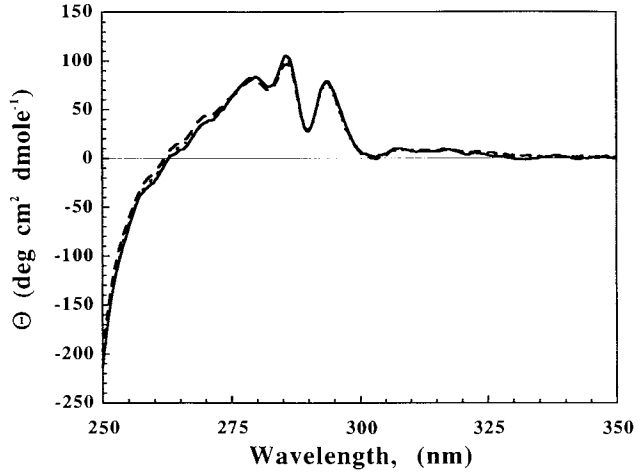

Fig. 2a. Near-UV CD of rhDNase using the Hudson T Up-Draft jet nebulizer. (——) Prior to nebulization; (--) nebulized aerosol; (-----) reservoir residual.

evidence that the pH change was unrelated to any chemical changes in the protein molecule.

CONCLUSIONS

rhDNase is one of the first recombinant DNA technology products indicated for delivery to the respiratory tract. Since jet nebulizers represent the simplest means of producing an aerosol in the respirable range, they were used to deliver rhDNase for this study. However, the recirculation of protein solutions under high shear rates in the nebulizer bowl could potentially damage the drug molecule (4,20).

This work shows that the four nebulizers tested are able to deliver 1 and 4 mg/mL aqueous solutions of rhDNase in respirable size particles. In addition, the rhDNase remains active and analytically unaltered (as tested by pH measurement, sizing chromatography, and near- and far-UV circular dichroism spectrophotometry) following aerosolization in all four devices.

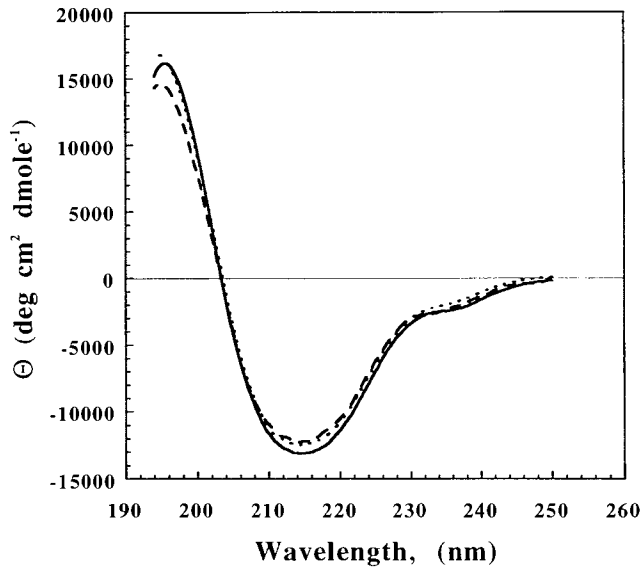

Fig. 2b. Far-UV CD of rhDNase using the Hudson T Up Draft jet nebulizer. (——) Prior to nebulization; (--) nebulized aerosol; (----) reservoir residual.

The analyses indicate that approximately 25% of the dose of rhDNase placed in the nebulizer was delivered in the respirable range of between 1 and 6 μm to the mouthpiece. These results are comparable to the maximum delivery of other solutes by jet nebulizers (22). All four devices were equivalent in their performance with the 4 mg/mL formulation. The two Marquest devices and the Hudson nebulizers were also found equivalent at delivering 1 mg/mL rhDNase. Given the relatively broad dose response curve observed for rhDNase in the clinical trials (2,22), a substitution of the Hudson jet nebulizer with the Baxter jet nebulizer is also most likely acceptable in terms of the near equivalence of performance in our laboratory tests.

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