ELSEVIER

International Journal of Pharmaceutics 109 (1994) 17–26

international journal of pharmaceutics

Protein nebulization: I. Stability of lactate dehydrogenase and recombinant granulocyte-colony stimulating factor to air-jet nebulization

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(Received 20 October 1993; Modified version received 20 January 1994; Accepted 27 January 1994)

Abstract

The effect of air-jet nebulization on the stability of two proteins was examined. Lactate dehydrogenase (LDH) and recombinant human granulocyte colony stimulating factor (rhG-CSF) were chosen as the test proteins. The enzymatic activity of LDH can be readily monitored using a colorimetric assay. rhG-CSF was also studied as it has potential to be delivered to the systemic circulation via the lung. Aerosolization of LDH using a three-jet Collison nebulizer resulted in a time-dependent loss of enzymatic activity. The extent of inactivation was dependent upon the applied air pressure and the volume of fluid in the nebulizer reservoir. Inactivation at 10 and 40 psig was 12 ± 5 ($n = 6$) and $18 \pm 10\%$ ($n = 6$), respectively, after a period of 10 min nebulization using a 40 ml starting volume, whereas an initial volume of 10 ml resulted in $57 \pm 14\%$ loss of activity after 10 min at 40 psig. rhG-CSF was affected in two ways by aerosolization. First, non-covalent aggregates were formed with time as observed by size exclusion chromatography. Second, a chemical change was induced and this was reflected in the formation of a higher mobility band on native gel electrophoresis. The change in mobility was thought to be due to an altered charge state of the protein, possibly through deamidation. Both the aggregated and degraded forms accounted for approx. 40% of the protein after 10 min. Polyethylene glycol (PEG) 1000 markedly reduced the deleterious effects of nebulization on the proteins. A 10 ml solution of protein containing 1% w/v PEG 1000 retained $\approx 100\%$ of the original activity of LDH after 10 min nebulization at 40 psig while both forms of rhG-CSF degradation were held to less than 10% for the same set of operating conditions.

Key words: Aerosol; Air-jet nebulization; Granulocyte-colony stimulating factor; Lactate dehydrogenase; Protein; Stability

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SSDI 0378-5173(94)00044-6

1. Introduction

Significant research is being focused on the lung as a target organ for topical therapy and as a portal of entry to the systemic circulation for protein and peptide therapeutics (Hubbard et al., 1989; O'Hagan and Illum, 1990; Vogelmeier et al., 1990; Aitken et al., 1992; Patton and Platz, 1992).The current devices available for the pulmonary delivery of these molecules are the pressurized metered dose inhaler (pMDI), dry powder inhaler (DPI) and the nebulizer. Each mode of delivery has the potential to adversely affect protein stability at some point in the formulation process or during actual aerosolization of the proteins. In particular, the formulation of proteins for pMDIs and DPIs presents a formidable challenge. The dehydration of the proteins and their subsequent comminution to produce powders of a size range suitable for inhalation therapy may result in a significant loss of biological activity. In comparison, there is a ready supply of proteins that have the immediate potential to be nebulized since they are invariably purified and initially formulated in aqueous solution for parenteral administration. As a consequence, nebulizers have already found utility as a means to deliver proteins to the lung. Hubbard et al. (1989) have shown that α_1-antitrypsin can be aerosolized without apparent loss of activity or changes in the protein structure. Other groups also have had measured success with secretory leukoprotease inhibitor (Vogelmeier et al., 1990), growth hormone (Patton and Platz, 1992) and DNase (Aitken et al., 1992). Unfortunately, these examples imply that proteins, in general, will survive aerosolization intact when in fact the opposite may be true.

Although solutions prior to nebulization may be stable, the process of aerosolization can potentially destabilize proteins via a number of mechanisms. First, the shear forces associated with the production of aerosol may be sufficient to affect some proteins directly. Denaturation of some proteins upon passage through filters has been attributed to shear effects (Charm and Wong, 1970). Second, a large air-water interface is continuously being produced within the nebulizer and the hydrophobic air component of this interface may result in surface denaturation of proteins (Andrews, 1991). A third source of instability could arise during the evaporation of droplets. The increase in protein concentration within the droplets and within the nebulizer reservoir fluid may ultimately destabilize the macromolecules.

In response to these issues, and since proteins are now being nebulized in a clinical setting (Aitken et al., 1992), we decided to undertake a study on the physical and chemical stability of two proteins to air-jet nebulization. Lactate dehydrogenase (LDH), a tetrameric enzyme, was chosen as a model protein since quantitative assays of its enzymatic activity are readily available. Recombinant human granulocyte-colony stimulating factor (rhG-CSF), a monomeric protein of M_r = 18800, was also studied, since it also has the potential to be administered by inhalation aerosol. Recent animal studies have shown it to elicit systemic activity when introduced to the lung by intratracheal instillation or by aerosol (Platz et al., 1992; Niven et al., 1993). These proteins were shown to undergo substantial time-dependent denaturation and degradation during air-jet nebulization. Attempts were then made to reduce denaturation using additives. From a choice of potential compounds, we initially examined PEG, since it is known to decrease the surface tension of water (Cooper and Eley, 1948), and be relatively inert to proteins (Arakawa and Timasheff, 1985; Bhat and Timasheff, 1992).

2. Materials and methods

2.1. Preparation of proteins

Lactate dehydrogenase (LDH; M4 isozyme from rabbit muscle) was purchased from ICN (Irvine, CA) and used without further purification. Prior to nebulization the crystalline suspension of LDH was dialyzed against 20 mM potassium phosphate buffer, pH 7.5 and diluted to a concentration of 25 μg/ml. Recombinant human rhG-CSF was produced using *E. coli* at Amgen Inc. (Thousand Oaks, CA) and formulated at 4 mg/ml in a 1 mM HCl buffer, pH 3.0.

2.2. Nebulization

Proteins were aerosolized using a Collison three-jet nebulizer. The body was machined from Delrin acetyl resin plastic (E.I. du Pont de Nemours and Co., Wilmington, DE) according to the specifications detailed by May (1973). For studies with LDH compressed air was generated from a portable compressor designed for inhalation therapy (Model DOL-101-AA, Gast Mfg. Corp., Benton Harbor, MI). This air was not dried prior to entering the nebulizer jets. Later studies with rhG-CSF used compressed air supplied from a gas cylinder (Linde Gas, Danbury, CT). This was dried through a 12 inch silica gel column (Natrasorb T silica beads (4 × 12 mesh); Multiform Desiccants Inc., Buffalo, NY) and filtered (HEPA capsule filter no. 12144; Gelman Sci., Ann Arbor, MI) prior to entering the nebulizer. The air entering the nebulizer had essentially zero relative humidity at room temperature.

The influence of time, applied air pressure and nebulization volume was examined with LDH solutions. Starting volumes of 10 and 40 ml were used at compressed air pressures of 10 (4.1), 25 (7.0) or 40 (10.0) psig (l/min). Nebulization was carried out for up to 60 min. However, the time of operation was dependent upon the volume in the reservoir. If 'sputtering' occurred in the nebulizer, then experiments were halted. The sputtering is due to air entrapment in the liquid feed inlets and occurs when there is insufficient fluid level to maintain an uninterrupted flow through the nebulizer jets. Samples of 100 μl were removed from the nebulizer reservoir fluid at fixed intervals during experiments. The temperature of solutions during nebulization was monitored via a temperature probe (8402–20, Cole-Parmer Inst. Co., Niles, IL). In these studies the output was not examined. This is complicated by the effects of droplet evaporation on the proteins.

2.3. Effect of PEG on protein stability

Certain excipients when added to protein solutions may exert a protective or destabilizing effect on the proteins during nebulization. Polyethylene glycol 1000 (PEG 1000; Carbowax Sentry 1000 NF, Union Carbide, Danbury, CT) was chosen for these studies because of its weak surface-active properties (Cooper and Eley, 1948) and its previously observed capacity to be excluded from the surface of proteins (Arakawa and Timasheff, 1985; Bhat and Timasheff, 1992). Solutions of rhG-CSF and LDH were nebulized as described with the addition of 1% w/v PEG 1000.

2.4. LDH activity assays

LDH enzymatic activity was measured at 25°C as follows. The 1.0 ml reaction mixture in 25 mM Tris-Cl buffer, 0.1 M KCl, pH 7.5 contained 2 mM pyruvate and 0.215 mM reduced nicotinamide adenine dinucleotide. The reaction was initiated by the addition of 5 μl of the LDH solution which had an enzyme concentration of 25 μg/ml. Activity was measured by the decrease in the absorbance at 340 nm using a Beckman DU 650 UV spectrophotometer with programmable temperature control (Beckman Inst., Inc., Allendale, NJ). The activity is expressed as the percentage of initial activity.

2.5. Polyacrylamide gel electrophoresis

For electrophoresis under native conditions a homogeneous 6% 1 mm thick Novex gel (Novex, Inc., San Diego, CA) was used in the absence of sodium dodecyl sulfate (SDS). The electrode buffer contained 25 mM Tris and 20 mM glycine. The sample for the gel contained the same concentrations of Tris and glycine, and 2.5% glycerol, 2.5% sucrose, and 0.025% β-bromophenol blue. A constant 50 V was applied to the gel, with a total running time of 7 h. Approx. 30 μg of rhG-CSF was loaded onto the gel and the gels were stained with Coomassie blue. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out using a Novex 4–20% gradient gel and 0.1% SDS in the electrode buffer. The sample also contained 1% SDS. The gels were run at constant 120 V. After destaining, the gels were subjected to densitometric analysis using an LKB Ultrascan XL laser densitometer (Pharmacia LKB Biotech Inc., Piscataway, NJ).

2.6. Size-exclusion column chromatography (SEC)

SEC experiments were run on a Superose 12 column (1 × 30 cm) (Pharmacia, Piscataway, NJ) using a mobile phase of 0.1 M sodium phosphate, pH 6.9 at 0.5 ml/min at 4°C. The column was connected to a Waters HPLC system (717 Autosampler, 510 pump, 490E UV detector ($A_{280\ nm}$); Waters Inst., Bedford, MA). Peak area was integrated and analyzed using Maxima 820 software (Dynamic Solutions, Bedford, MA) on an IBM PC. The eluents were also followed by laser light scattering and a differential refractometer in order to determine the molecular weights of the proteins as described previously (Philo et al., 1993).

3. Results

3.1. Nebulization of LDH and rhG-CSF

Aerosolization of the LDH at all operating conditions results in an irreversible time-dependent loss of enzymatic activity in the reservoir solution. On average, about 20% of the initial activity is lost after 10 min nebulization at 40 psig and a starting volume of 40 ml (Table 1), while the activity loss increased to 65% at 60 min nebulization (Fig. 1). The loss of activity is reduced as the applied air pressure is reduced, but not dramatically (Table 1 and Fig. 1). However, a change in the initial fluid volume in the reservoir from 40 to 10 ml results in a substantial increase in the rate at which the enzyme is denatured and inactivated. After 10 min, 43 ± 14% remains compared with 82 ± 10% ($n \geq 5$) using a 40 ml starting volume. In all cases, the inactivation of LDH appears to follow first-order kinetics. An example of such plots is shown in Fig. 2. The rate of inactivation is dependent upon the operating conditions (Table 1).

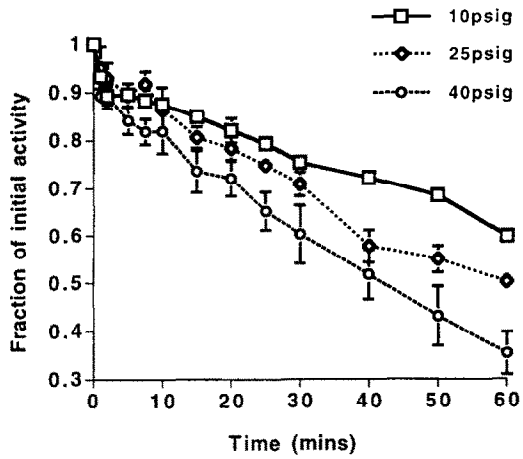

Fig. 1. Effect of air pressure (psig) on LDH activity during nebulization. Activity is lost as a function of time and this phenomenon is exacerbated as the air pressure is increased [10 (□), 25 (◇), 40 (○) psig]. The mean results of ≥ 5 experiments are shown for each air pressure. Error bars are the standard error of the mean.

Table 1
Effect of operating conditions and PEG1000 on LDH activity

Condition	k [a] (×10^{-2})	% activity remaining [b]
Air pressure [c] (psig)		
10	0.7	87.6
25	1.1	82.9
40	1.6	82.0
PEG 1000 [d] (%, w/v)		
0	8.8	39.4
0.01	4.6	68.8
0.1	2.1	81.9
1	0.6	92.6

[a] k, apparent first-order rate constant (min^{-1}) of inactivation.
[b] % activity remaining after 10 min nebulization.
[c] In this series of experiments, a starting volume of 40 ml was used.
[d] In this series of experiments, a volume of 10 ml and an air pressure of 40 psig were used.

In subsequent experiments with rhG-CSF, a starting volume of 10 ml and a 40 psig pressure were employed. These operating conditions produced the most severe denaturation of LDH and are not unlike those that may be employed in the clinic. rhG-CSF has been shown elsewhere to be relatively unstable at pH above 7.0 (Oh-eda et al., 1990; Arakawa et al., 1993). Nebulization of the protein was therefore carried out at an acidic pH, primarily in unbuffered 1 mM HCl at pH 3.0. Under these conditions, rhG-CSF is stable at 4°C for a prolonged period (Arakawa et al., 1993). Nebulization of rhG-CSF in this solvent at 4.0

mg/ml results in some foaming within the reservoir but upon completion of experiments a clear solution remains after settling. The stability of rhG-CSF during nebulization was examined using SEC at 4°C in 0.1 M sodium phosphate at pH 6.9, since the elution of protein from SEC in an acidic environment was observed to be poor and irreproducible. The use of 0.1 M sodium phosphate was further justified for the following reasons. Firstly, injection of a starting G-CSF solution onto the column gave a single peak suggesting that SEC did not adversely affect the state of rhG-CSF. Secondly, when a sample of nebulized rhG-CSF at 4.0 mg/ml was injected onto the column, an elution profile similar to that shown in Fig. 3 was observed. A similar profile was observed when the same G-CSF solution was first diluted 10-fold with PBS and then injected onto the column. This suggests that prior dilution of the acidic samples with a neutral buffer is unnecessary. Thirdly, the aggregation state observed by SEC in 0.1 M sodium phosphate is a more practical demonstration of the environment that rhG-CSF would be exposed to upon inhalation.

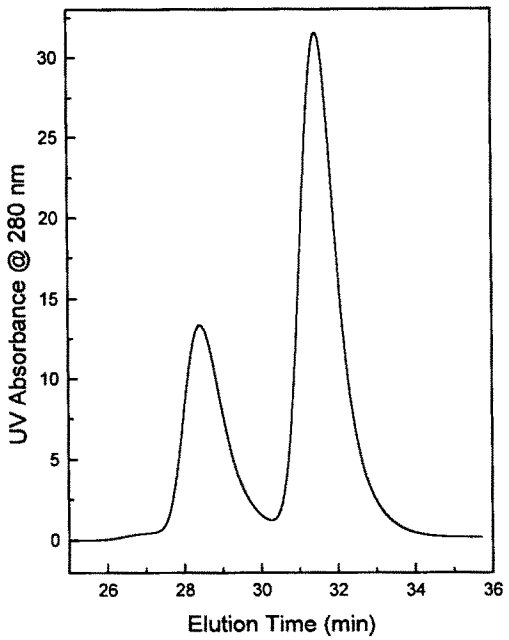

Fig. 3. SEC chromatogram of rhG-CSF after nebulization (10 min, 40 psig). Chromatographic conditions: Superose 12 column (1×30 cm); mobile phase, 0.1 M sodium phosphate pH 6.9; flow rate, 0.5 ml/min at 4°C.

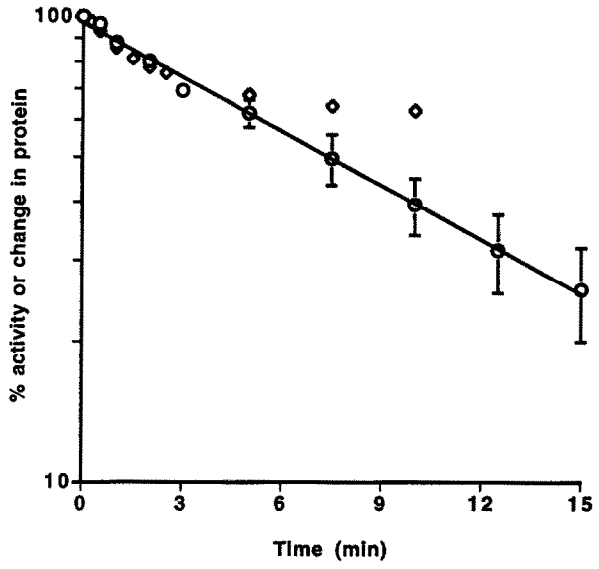

Fig. 2. Semi-log plot comparing the percentage decline in activity of LDH (○) and the percentage decline of rhG-CSF monomer (◇) during nebulization. The line is the best fit by least-squares regression through the LDH data. The nonlinearity of the decrease in rhG-CSF monomer is clearly observed.

All the nebulized rhG-CSF samples demonstrate a major new peak eluting 3 min earlier than the peak corresponding to the unnebulized sample (Fig. 3). Laser light scattering analysis of this peak revealed it to be a dimer of rhG-CSF, and as expected, the main peak is a monomeric form. Colony forming activity of the isolated aggregated form was about 70% of the starting material, whereas the main peak for the monomer showed an activity indistinguishable from that of the starting material. Since the major aggregation product is the dimer, the nebulization results for rhG-CSF are expressed as a percentage of that form in the total integrated area of the eluting peaks.

The time dependence of dimer formation was first examined. The fraction of dimer increases with the time of nebulization and is visibly present in samples removed 15 s after initiation of aerosolization. After 5–10 min nebulization, the

aggregate formation approaches a plateau that typically accounts for 35–40% of the total integrated peak area (Fig. 4). The changes in rhG-CSF do not follow the simple exponential decline in activity observed with LDH (Fig. 2) and this implies a more complicated mechanism of denaturation.

rhG-CSF is also observed to be denatured in a second way. Samples run on polyacrylamide gels under native conditions, with no SDS present, show the presence of a new band migrating slightly faster than the intact protein. This new band is clearly present in samples removed after 2 min aerosolization (Fig. 5). The intensity of the new band increases with time of nebulization as determined from densitometric analysis but, as with the aggregate, appears to saturate after approx. 10 min operation (Fig. 6). It would ultimately be interesting to compare the kinetics of degradation with that of aggregation. However, the inaccuracy of densitometric analysis may preclude this possibility. The native gel does not show the presence of the aggregate. It presumably dissociates during the electrophoresis due to the high operating pH (8.6).

To determine if aggregate formation was covalently linked or if the degradation was due to peptide cleavage, the nebulized samples were also analyzed by non-reducing SDS-PAGE. Only a single band was observed on a Coomassie blue stained gel (Fig. 5). The results demonstrate that aggregation is due to a non-covalent interaction and that degradation observed using native PAGE

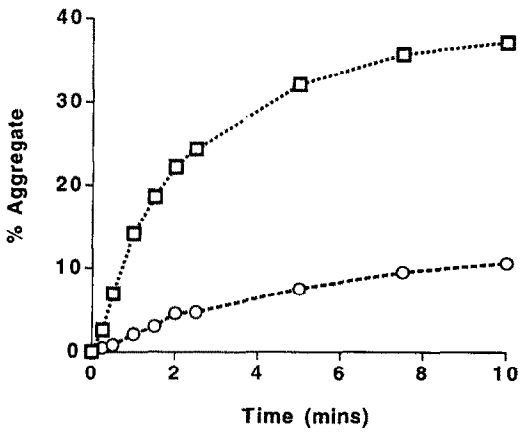

Fig. 4. Percentage of aggregated rhG-CSF as a function of time of nebulization (40 psig, 10 ml) in the presence (○) and absence (□) of 1% w/v PEG 1000.

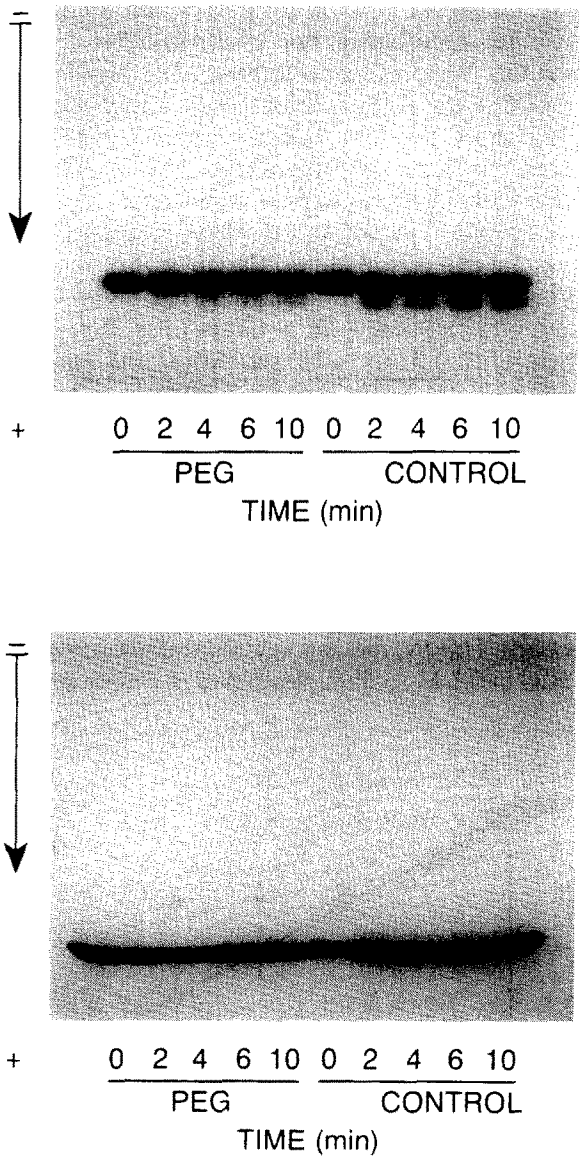

Fig. 5. Photograph of a native polyacrylamide gel electrophoresis (upper) and SDS-PAGE (lower) illustrating the degradative effects of nebulization in the absence and presence of 1% w/v PEG. A new band appears on the native gel that increases in intensity with time of nebulization but is diminished in the presence of PEG. The absence of any additional band on the SDS gel suggests that no change in the molecular weight of rhG-CSF is caused by nebulization.

does not necessarily involve a change in molecular weight. A change in the charged state of the protein is most likely, possibly brought about by deamidation, although this has yet to be confirmed.

3.2. Effect of PEG on protein stability

Nebulization of LDH solutions containing 1% (w/v) PEG 1000 at 40 psig and a 10 ml starting volume results in almost complete retention of enzymatic activity over a 20 min period of aerosolization. This protective effect is, however, a function of the PEG concentration and is reduced as the PEG concentration is reduced (Fig. 7). Nevertheless, some protection is conferred at concentrations as low as 0.01% (w/v) (Fig. 7).

PEG 1000 also stabilizes 4 mg/ml rhG-CSF solutions during nebulization. A marked reduction in aggregate formation (Fig. 4; 11% vs 37% without PEG 1000 after 10 min) occurs. The intensity of the second band on the native gel is also markedly reduced where PEG 1000 has been added to the nebulizing solution (Fig. 5 and 6). SDS-PAGE of these nebulized samples in the presence of PEG 1000 gives a single band. A similar concentration dependence of PEG is observed for rhG-CSF. At 0.01% (w/v) PEG a lower but significant level of stabilization is observed. After 10 min of nebulization, 32% aggregate formation is observed vs 37% in the absence of PEG.

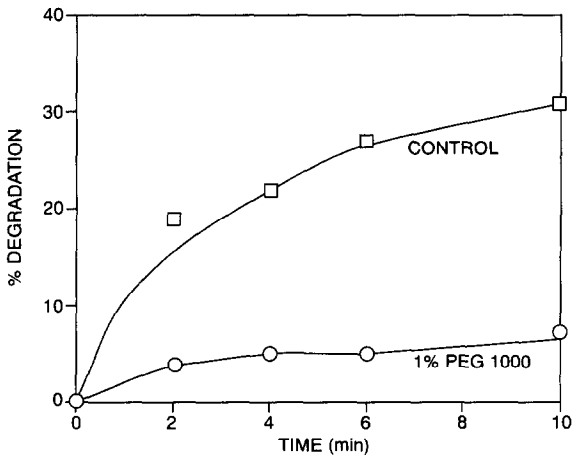

Fig. 6. Percentage of degraded rhG-CSF as a function of time of nebulization (40 psig, 10 ml) in the presence (○) and absence (□) of 1% w/v PEG 1000. The similarity between the degradation and aggregation profiles (Fig. 4) suggests that the two effects are interrelated.

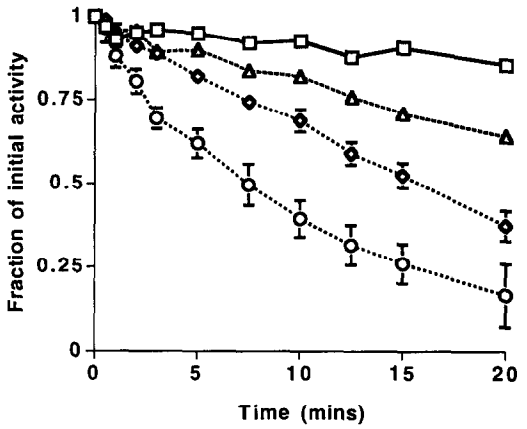

Fig. 7. Influence of PEG 1000 concentration on LDH activity during nebulization (40 psig, 10 ml). A marked decrease in the loss of activity is observed as the PEG concentration is increased [0 (○), 0.01 (◇), 0.1 (△), 1 (□) % w/v]. The mean results of five experiments are shown for each PEG concentration except 0.1% w/v where $n = 1$. Error bars are the standard error of the mean.

4. Discussion

These results have demonstrated that two proteins, one with potential for clinical use as an inhalation aerosol, can be denatured by air jet nebulization. It is therefore pertinent to discuss the possible mechanisms of denaturation that take place during air-jet nebulization.

As alluded to earlier, there are several potential causes of protein denaturation during nebulization: (1) shear stress, (2) surface effects and increased potential for chemical reactivity, and (3) protein concentration in evaporating water droplets. These three effects occur simultaneously during nebulization and are interrelated. This makes it difficult to identify the relative contribution of each to the observed denaturation of rhG-CSF and LDH. Other less obvious mechanisms may also contribute to the observed denaturation.

As air pressure is increased, the inactivation of LDH is accelerated. However, this does not necessarily imply that this result is entirely due to increased shear stress. The aspiration rate of liquid through the nebulizer jets is also increased. Hence, more aerosol is being produced per unit time and therefore the result also may reflect an increase in surface denaturation. In addition, some decrease in droplet size occurs with increasing air pressure and although this implies an increase in the shear force required to produce the smaller droplets it also indicates greater production of new surface area.

The introduction of additives, such as PEG, to the nebulizer solutions have the potential to physically alter the size distribution of aerosol through changes in surface tension and viscosity (Nukiyama and Tanasawa, 1939). Thus, the quantity of new surface produced over time may be affected. However, the median droplet size and polydispersity of the aerosol that is produced within the nebulizer is unknown. It should be borne in mind that only a small fraction of the aerosol that is produced actually escapes as inhalable droplets. So it is difficult to quantify the effects of additives on droplet size unless measurements are made directly on the spray emerging from the nebulizer jets.

The effect of concentration of protein within the droplets was not examined in this study and this may have a contributory destabilizing effect on protein that ultimately reaches the lungs of a patient. The degree of concentration also will be very much dependent upon the nebulizer operating conditions. Factors, including the length of exit tubing, velocity of aerosol, temperature, relative humidity, initial concentration and initial droplet size will all influence the protein concentration in droplets prior to them entering the oral cavity. It is not known if instability arising from concentration of protein is likely to produce an instantaneously observable effect. The time between generation of aerosol and inhalation is probably of a sufficiently short duration that significant damage is avoided. However, further work on the role of concentration on the stability of aerosolized proteins is needed.

The observed protein denaturation is most likely associated with the generation of a large air-water interface within the nebulizer. Exposure at interfaces can induce conformational changes in protein structure (Andrews, 1991). In the case of LDH this may be an irreversible change that affects the epitope(s) associated with enzymatic activity. With rhG-CSF, the tertiary structure may be transiently opened exposing sites that enable physical aggregation, as well as chemical changes. The close proximity of individual protein molecules at the surface also may provide favorable conditions for aggregation to progress.

The validity of these suppositions rely on the assumption that a significant portion of the protein will be exposed to new surface over the period of nebulization. This seems reasonable, since we can estimate that some 1500 m^2 of surface will be produced over 10 min of operation assuming a 10 ml fluid volume, 10 μm median droplet size and an aspiration rate of 100 ml/min through the nebulizer jets. In addition, the time of exchange of protein between the surface and bulk of a droplet will probably be substantially shorter than the lifetime of the droplet, thus increasing the probability of surface exposure.

One noticeable difference between the loss of activity of LDH and the changes that take place in the rhG-CSF is in the extent of the damage. The physical and chemical changes in rhG-CSF appear to be self-limiting and plateau at 30–40% of the total protein present. In comparison, the rate at which LDH loses activity appears constant and most is lost by the end of the nebulization experiments. It is not known why these differences occur. One plausible hypothesis is that the aggregation induced in the rhG-CSF may hinder further molecules from reaching the air interface. As this increases, the chances of monomer being exposed will be reduced. It is also possible that the denaturation reaches an equilibrium state, and aggregation may be a reversible process. The LDH studies also were performed at a much lower concentration at which the surface concentration is probably not saturated.

Polyethylene glycol was shown to stabilize both LDH and rhG-CSF. The stabilizing effect of PEG

on rhG-CSF is of practical importance, considering potential clinical use of rhG-CSF when administered as an inhalation aerosol. One possible mechanism that confers stability may relate to the weak surfactant properties of PEG. It may prevent denaturation by competing with the proteins for the generated surface. This implies that the protection is concentration dependent and this was confirmed by nebulizing LDH in various PEG 1000 concentrations (Fig. 2). It is worth noting that PEG in itself does not adversely influence protein stability. Arakawa and Timasheff (1985) have shown that PEG is preferentially excluded from the surface of proteins. Many other surface active agents, although capable of lowering surface tension to a greater extent than PEG, may bind to the surface of proteins (Tanford, 1980; Hjelmeland, 1986). In addition, PEG has little effect on the thermal denaturation of proteins.

These studies have demonstrated that proteins can be adversely affected by air-jet nebulization. Any protein being considered for inhalation therapy should be thoroughly investigated for its stability to aerosolization prior to any clinical use. If not, reduced efficacy and increased toxicity may result. Less intact bioactive protein may deposit in the lung than expected and deposition of aggregated protein may predispose toward immunogenicity. The use of excipients that minimize or prevent instability, and which are also nontoxic, are required. In this regard PEG 1000 or other PEGs of different molecular weights may be acceptable. Further work is needed to elucidate the mechanisms of protein instability as well as the mechanisms of protection.

Acknowledgements

The authors wish to thank John Philo and Jie Wen for performing the light scattering and differential refractometry analyses and Joan Bennett for preparation of the manuscript.

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