Lyophilized Formulations of Recombinant Tumor Necrosis Factor[1]

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Recombinant tumor necrosis factor-alpha (TNF), an investigational biological response modifier, is a protein and is susceptible to particulate generation during handling in dilute aqueous solutions. TNF is prone to formation of nonreducible dimers and oligomers during formulation, lyophilization, and storage. The effect of various parameters, such as the pH, protein concentration, and nature of excipients present during lyophilization, on the formation of nonreducible dimers and oligomers was investigated. The results of these studies indicate that these parameters can significantly alter the rate of this reaction. Inclusion of an amorphous buffer and an appropriate amount of a crystallizing sugar (mannitol) combined with a suitable quantity of an amorphous protectant (dextran, sucrose, trehalose, or 2-hydroxypropyl-β-cyclodextrin) was shown to reduce the formation of these dimeric and oligomeric species during lyophilization. Representative lyophilized formulations of TNF based on selected amorphous excipients were found to be fully bioactive and stable over 9 months.

KEY WORDS: tumor necrosis factor; protein formulations; lyophilization of proteins; amorphous protectants; nonreducible oligomers.

INTRODUCTION

Tumor necrosis factor (TNF) is a biological response modifier with demonstrated cytotoxic activity against various murine and human cell lines (1). Native TNF protein is synthesized and secreted by macrophages (2). Recombinant forms of TNF, such as TNF-α (TNF) derived from *Escherichia coli,* essentially retain the biological activity of the native molecule (3,4) and are under investigation for their therapeutic effects in various cancers and infectious diseases (5). Being a potent cytokine, TNF is used in very small quantities for biological action. This makes it susceptible to particulate generation during handling in aqueous solutions. It was, therefore, considered desirable to formulate this protein in a freeze-dried form. The molecular mass of human TNF has shown to be 17 kDa, but it exists as a compact trimer in aqueous solution (6–8) and is prone to nonreducible dimer formation during the process of formulation. The present investigations were undertaken to develop a stable, lyophilized formulation of TNF.

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MATERIALS AND METHODS

TNF and Excipients

Highly purified TNF from *E. coli* was prepared at Cetus Corporation (9,10). Dextran (molecular weight 79,000, clinical grade; Sigma Chemicals), polyethylene glycol (molecular weight 8000; Union Carbide Corporation), and 2-hydroxypropyl-β-cyclodextrin (Pharmatec, Inc.) were used as excipients. All other chemicals used were of USP/NF or ACS analytical reagent grade.

Formulation and Lyophilization

Purified TNF was desalted into the appropriate buffer medium by dialysis or gel filtration and combined with solutions of other excipients with gentle stirring. This formulated TNF was dispensed into 3-ml Type I glass vials with 13-mm lyophilization stoppers. Lyophilization was carried out in a Lyolab G freeze-dryer (LSL, Secfroid, SA). The vials containing TNF solution were frozen at −50°C for 4 hr, followed by sublimation for 18 hr at −30°C and 35-μm pressure and then drying at 15°C for 8 hr at the same pressure. Vials were stoppered under nitrogen and stored at various temperatures. At preselected intervals, TNF samples were reconstituted with 1 ml water for injection (WFI) and characterized.

Biological Activity

TNF bioassays were performed in 96-well microtiter plates. Actinomycin D-treated murine L929 cells were exposed to serially diluted TNF preparations (9). Cell cytotoxicity was determined by measuring the number of viable cells with the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide dye, MTT (11). One unit of an in-house recombinant TNF standard was defined as the reciprocal of the dilution required to kill 50% of the cells. All bioassays were run in parallel with an in-house standard, and activities are expressed as laboratory units.

Oligomer Formation

Quantitative measurement of TNF protein purity was carried out by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 12–15% linear gradient polyacrylamide gel, as described by Laemmli (12). Samples were heated to 95°C in a glycerol/SDS solution either with or without 2-mercaptoethanol. Protein samples (7 μg per lane) were applied and subjected to electrophoresis at 20 mA current for approximately 2 hr. Protein was visualized by incubation with Coomassie blue (0.1%) in water–methanol–acetic acid (45:45:10 by volume). Gels were scanned with a Camag Thin-Layer Chromato Scanner II, and the peaks integrated with a Nelson 6000 data system using an exponential skim aligorithm.

RESULTS AND DISCUSSION

Preformulation Studies

Preliminary experiments indicated that highly purified

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TNF is extremely susceptible to formation of cross-linked aggregates. These crosslinked aggregates consist of dimers, trimers, and higher oligomers. Further, SDS-PAGE analyses of TNF samples treated with β-mercaptoethanol indicated that these cross-linked species were nonreducible. No other significant changes in the TNF protein were observed during these preliminary studies. Thus, formation of nonreducible dimers and oligomers of TNF was considered the most sensitive parameter for preformulation and formulation studies. In preformulation studies, we examined the effect of various parameters on the stability of lyophilized TNF: the pH, protein concentration, and nature of the other excipient components.

Effect of pH

TNF solutions containing 0.25 mg/ml of protein, 1% mannitol, and an appropriate buffer (10 m*M* of sodium citrate, phosphate, or borate) were prepared at pH values of 4, 6, 8, and 10. After lyophilization, these samples were placed at 37°C for a month. The results are presented in Table I. As shown, nonreducible dimers were not formed at lower pH values (4 and 6). At pH 4, however, a drop in the bioactivity of TNF was observed. These data indicate that, for TNF, the pH of maximal stability under these conditions is close to 6. A limitation of this evaluation was that different buffer systems, which may have been partly responsible for some of the observed changes, were employed for pH adjustment of TNF solutions.

Protein Concentration

The effect of TNF concentration on the rate of nonreducible oligomer formation during storage of the lyophilized product was also evaluated. One-milliliter aliquots of formulations containing 0.05–2.5 mg/ml TNF and 1% mannitol in 10 m*M* sodium phosphate (pH 7.0) were lyophilized. These preparations were placed at 37°C for 2 weeks, reconstituted with 1 ml WFI, and characterized. The data from these studies are presented in Fig. 1. As the concentration of TNF increases, there is a corresponding decrease in the percentage of nonreducible dimers. However, expressed as micrograms of nonreducible dimers, the curve is the reverse, with a plateau around 6 μg, for a TNF concentration of approximately 1 mg/ml. It appears that during lyophilization, only a certain amount of TNF is exposed to destabilization and consequently susceptible to nonreducible dimer formation.

Table I. Effect of pH on the Stability of Lyophilized TNF

pH	Buffer (10 m*M*)	After 1-month storage at 37°C: % nonreducible dimer	After 1-month storage at 37°C: Bioactivity ($\times 10^7$ U/mg)
4.0	Na citrate	0.0	0.3 ± 0.1
6.0	Na citrate	0.0	1.0 ± 0.2
7.4	Na phosphate	5.4	0.8 ± 0.2
8.0	Na phosphate	6.2	0.9 ± 0.2
10.0	Na borate	10.0	0.8 ± 0.3

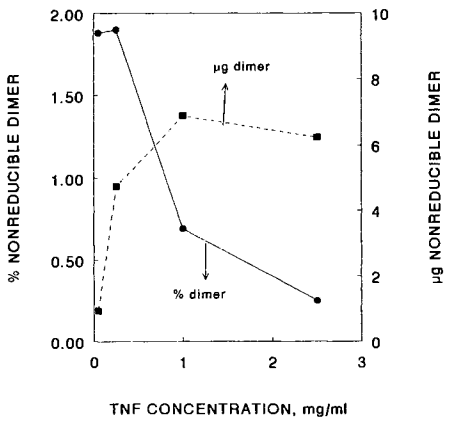

Fig. 1. Effect of TNF concentration on the generation of nonreducible dimers in lyophilized TNF preparations. The data are presented as both percentage and total micrograms of nonreducible dimer formed.

Nature of Excipient Used

Bulking agents are commonly used in formulations to increase the amount of solids present after lyophilization, providing either a crystalline or an amorphous matrix in which the drug is freeze-dried. The physicochemical reactions that characterize the behavior of various solutes during freezing and freeze-drying have been discussed in detail in the literature (13,14). It is known that, during freezing, any crystalline solute present in the formulation crystallizes in a manner that is dependent upon the thermal properties of the mixture. It has also been hypothesized that proteins behave as amorphous substrates and tend to form protein-rich aqueous concentrates interspersed between ice crystals. Amorphous excipients form part of the protein-rich glassy concentrate. These behaviors can have important implications regarding stability of proteins during freezing, freeze-drying, and storage of the freeze-dried product.

In view of the above information, the effect of excipient type (i.e., amorphous vs crystalline) on the formation of the nonreducible dimer during lyophilization of TNF was studied. Representative results are presented in Table II. TNF was lyophilized in the presence of mannitol, a crystallizing solute, and either sodium phosphate or sodium citrate

Table II. Amorphous Versus Crystalline Excipients for Lyophilized TNF Preparations[a]

Amorphous component	After 1-month storage at 37°C: % nonreducible dimer	After 1-month storage at 37°C: Bioactivity ($\times 10^7$ U/mg)
None	0.00	1.2 ± 0.2
None	1.46	1.2 ± 0.3
2% dextran	0.00	1.5 ± 0.2
0.5% sucrose (1)	0.00	1.2 ± 0.3
2.0% trehalose	0.20	1.8 ± 0.5
0.5% HPCD	0.10	1.6 ± 0.3
2.0% PEG-6000	0.00	1.0 ± 0.2

[a] All formulations contained TNF at a concentration of 0.25 mg/ml, 1% mannitol, and 10 m*M* sodium citrate, pH 6.5. The concentration of mannitol in the formulation (1) was 1.5%.

buffer. Inclusion of sodium phosphate buffer, which is known to exhibit differential crystallization behavior during freezing (15,16), enhanced oligomer formation (data not shown). An amorphous buffer, such as sodium citrate, decreased but did not always eliminate the dimer formation in our test protocol. However, when amorphous solutes, such as dextran, sucrose, trehalose, 2-hydroxypropyl-β-cyclodextrin (HPCD), or polyethylene glycol-6000, were employed for formulating TNF in combination with mannitol, essentially no dimer formation was observed. All these formulations were also bioactive, as shown in Table II.

Formulation Studies

From preformulation studies, it was concluded that the following characteristics were desirable for stabilizing TNF in a lyophilized formulation: (a) pH close to 6 using an amorphous buffer system, (b) TNF concentration of 1 mg/ml or more, and (c) an amorphous bulking agent. Sodium citrate was chosen as the amorphous buffer and the pH was fixed at 6.5 to be in the near-physiological range. Since TNF is a potent molecule, a concentration of 0.25 mg/ml was satisfactory for clinical use. For the bulking agent, we considered the use of a mixture of a crystalline and an amorphous solute. We envisioned that such a mixture would yield good cake properties upon lyophilization (due to the crystalline part) while maintaining adequate stability of TNF (due to protection from the amorphous component). Following this strategy, we selected two formulations having the same buffer (10 m*M* sodium citrate, pH 6.5) and the crystalline excipient (10 mg/ml mannitol) but containing different amorphous protectants: 20 mg/ml dextran (DMC formulation) and 5 mg/ml sucrose (SMC formulation). A third formulation containing no amorphous excipient was also run as a control; this was based on a sodium phosphate buffer (10 m*M*, pH 7.5) (MP formulation).

These formulations were evaluated for stability upon storage under different temperature conditions. Stability of TNF in these formulations was monitored using the following parameters: biological activity by the cell cytotoxicity assay and protein purity by SDS-PAGE. No significant change in the bioactivity of TNF was observed in any formulation over a period of 9 months (273 days) at the temperatures studied. Nonreducible dimer formation in DMC, SMC, and MP was monitored by the reducing SDS-PAGE procedure. Data are presented in Table III. All formulations were completely free of nonreducible oligomers at −20 and 2–8°C storage over 9 months (data not shown). At 25 and 37°C, measurable amounts of oligomeric protein were detected in all formulations, as shown. The control formulation MP, which contained no amorphous excipient, exhibited the formation of significant amounts of nonreducible dimer at these temperatures. Data from studies on dextran- and sucrose-containing formulations were insufficient to allow for extensive kinetic evaluation.

Table III. Nonreducible Dimer Formation in DMC and SMC Formulations

Storage temperature	Storage period (days)	Percentage dimer in formulation		
		MP	DMC	SMC
Initial	—	0.00	0.00	0.00
25°C	91	0.80	0.00	0.00
	179	2.00	0.26	0.24
	273	1.80	0.50	0.78
37°C	42	2.42	0.19	0.00
	91	4.53	0.57	0.16
	150	4.25	0.56	—
	179	7.70	—	0.20
	273	11.40	0.88	0.48

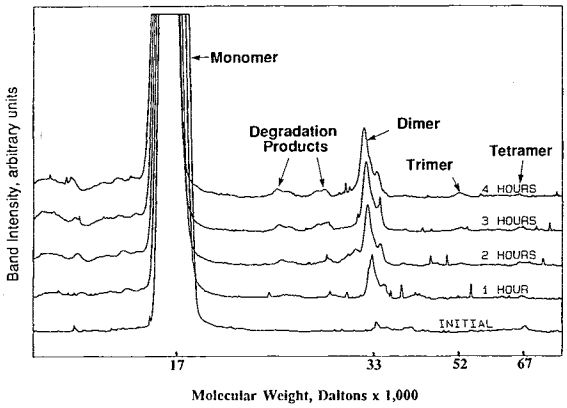

Fig. 2. Densitometric scan of an SDS-PAGE analysis of a MP formulation sample incubated at 80°C for specified periods of time: band density (as arbitrary units) is plotted against molecular weight (as daltons) on the scanned gel. Scans of samples subjected for different time durations (1 to 4 hr) are overlaid for comparison. The position of various species are indicated.

To accelerate formation of the nonreducible dimer, SMC was subjected to short incubations at 55, 65, and 80°C. Formulation MP was again run as a control. Nonreducible dimers and higher oligomers were monitored. Several species were formed in MP, as shown in Fig. 2, which displays the densitometric scan of a SDS-PAGE result from the 80°C study. These included dimer, trimer, and two peaks corresponding to a molecular weight between the monomer and the dimer. The two later peaks are probably a result of degradation of the dimer and the trimer. In contrast to these results obtained from MP, results with SMC indicated that

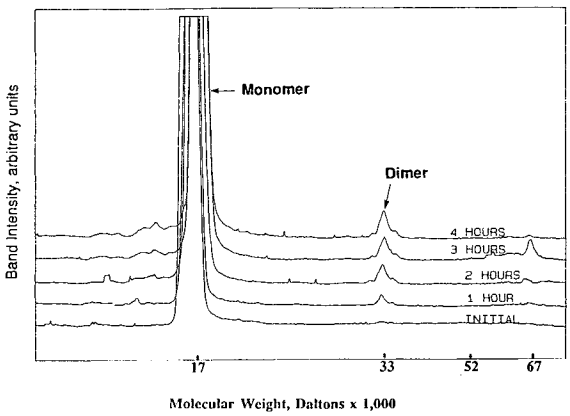

Fig. 3. Densitometric scan of an SDS-PAGE analysis of SMC formulation. Sample incubated at 80°C for specified periods of time. See the legend to Fig. 2 for further details. The peak at a molecular weight of 67,000 at the 3-hr time point is probably an artifact.

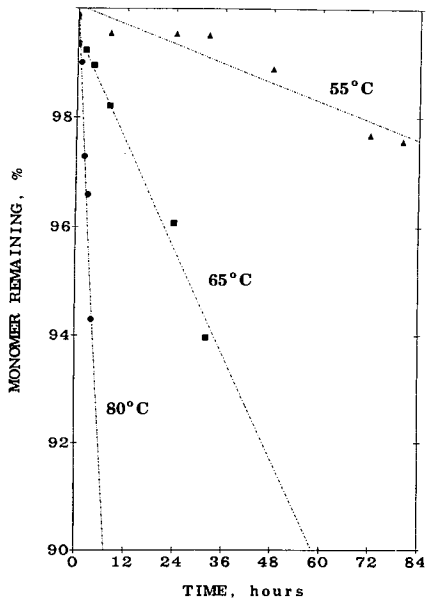

Fig. 4. TNF monomer remaining (as % of initial) in MP formulation at 55, 65, and 80°C, as a function of the time of incubation (hours).

fewer species of TNF were formed in this formulation. A typical densitometric scan is presented in Fig. 3.

Next, the amount of TNF monomer remaining in the MP formulation was plotted as a function of the time of incubation at the three temperatures (Fig. 4). Figure 5 presents similar data for the SMC formulation. It is evident from these data that loss of the monomeric form of TNF is much more rapid in the absence of an amorphous excipient in the formulation. Arrhenius plots of these data yielded activation energies for nonreducible oligomer formation of 33.8 and 41.2 kcal/mol for MP and SMC formulations, respectively. These results demonstrate that inclusion of an amorphous excipient, such as sucrose, enhances the energy barrier for formation of nonreducible oligomers of TNF.

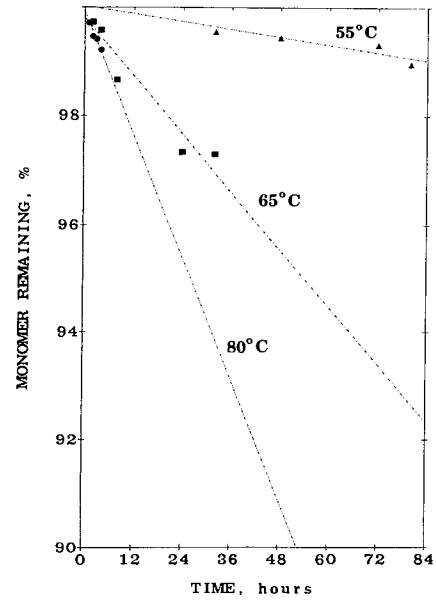

Fig. 5. TNF monomer remaining (as % of initial) in SMC formulation at 55, 65, and 80°C, as a function of the time of incubation (hours).

In conclusion, we have shown that inclusion of an amorphous buffer and an appropriate amount of a crystallizing sugar (mannitol) combined with a suitable quantity of an amorphous protectant (dextran, sucrose, trehalose, or 2-hydroxypropyl-β-cyclodextrin) in the TNF solution before lyophilization results in formulations that are stable for at least 9 months at refrigeration temperatures, as measured by bioactivity and protein purity.

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