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Preparation and Characterization of Liposomal-Lipophilic Tumor Necrosis Factor[1]

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ABSTRACT

The liposomal delivery of recombinant human tumor necrosis factor (rHuTNF) has been shown to be effective in reducing its toxic effects and in its targeting of organs rich in cells of the reticuloendothelial system. However, native recombinant human TNF shows only poor affinity for liposomes, presumably due to its low hydrophobicity. In an attempt to increase the efficiency of association with liposomes, we have modified TNF to increase its hydrophobicity by selective substitution of its amino groups with fatty acid side chains. *N*-Hydroxysuccinimide esters of saturated fatty acids ranging from C_8 to C_{18} were reacted with recombinant human TNF. Modification with esters of C_8 to C_{14} acids occurred as determined by consumption of positively charged amino groups monitored by native polyacrylamide gel electrophoresis; however, esters of longer chain lengths (C_{16}, C_{18}) were much less capable of introducing these chains via amide linkages, and thus these adducts were not further characterized. Biological assays revealed that retention of activity was inversely dependent both on the number of chains introduced and on their length; activity was most conserved (>50%) in a TNF preparation modified with only ~1-2.5 caprylic acid (C_8) residues/trimer. This preparation was found to bind with high efficiency (~50%) to preformed dipalmitoylphosphatidylcholine-small unilamellar vesicles. The extent of binding closely paralleled both the number of chains introduced and their length; binding was even more efficient (80-90%) for TNF modified either with ~3.5 caprylic acid residues/trimer or with ~1.5 residues of myristic acid (C_{14}). However, the biological activity of these acylated TNFs was further reduced by this more extensive chemical modification (<50% activity for C_8 and <10% for C_{14}). The biological activity of dipalmitoylphosphatidylcholine-small unilamellar vesicle-C_8-TNF was found to be comparable to that of the nonliposomal C_8-TNF. Thus, biologically active preparations of liposomal-lipophilic TNF can be prepared with high efficiency.

INTRODUCTION

TNF[3] has been the subject of initial evaluation in phase I/phase II clinical trials at institutions worldwide (1-4). It is clear from this evaluation that the major toxicity and impediment to further development is dose-limiting hypotension (1), perhaps due to direct effects on vascular endothelium (5-8). A strategy to better localize this cytokine in the tumor microenvironment and diminish its systemic accessibility to normal tissue would be valuable.

Liposomes are emerging into early clinical evaluation as nontoxic drug carriers. They appear to be particularly suited as carriers for hydrophobic drugs, and pharmacokinetic models suggest that lower systemic drug levels should be achieved with a drug-carrier formulation, resulting in reduced toxicity. At the same time, liposomes may target drugs directly to reticuloendothelial cell-rich organs, such as lung and liver, and indirectly to tumor beds via reticuloendothelial system-mediated trafficking (reviewed in Ref. 9). The latter may be particularly effective for TNF, inasmuch as some reports indicate that intratumoral administration is the most effective route (10).

Initial studies of liposomal-TNF formulations have been encouraging in that they have demonstrated tumor cytotoxicity *in vitro* and reduced tissue damage and better liver and lung targeting *in vivo* by these preparations compared to free TNF (11, 12). However, native TNF demonstrated low encapsulation or association efficiencies for the liposomes used in these studies; *e.g.*, it bound with an efficiency of only 3.9% to preformed multilamellar vesicles of phosphatidylglycerol/cholesterol.

In this paper, we have attempted to overcome this problem by increasing the hydrophobicity of rHuTNF through chemical modification. An increase in hydrophobicity may both substantially enhance the efficiency of the formulation process and, perhaps even more importantly, stabilize the liposome-TNF (11, 12) interaction to prevent undesirable dissociation *in vivo*.

MATERIALS AND METHODS

Materials

rHuTNF was obtained as a generous gift from Bio-Gen Corp. (Cambridge, MA). The fatty acids, *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, DPPC, and dimethyl sulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO). IODO-GEN was obtained from Pierce Chemical Co. (Rockford, IL). PD-10 columns were obtained from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Carrier-free $Na^{125}I$ was purchased from Amersham Corp. (Arlington Heights, IL). L-929 cells were obtained from the American Type Culture Collection (Rockville, MD).

Methods

Radioiodination of TNF. Purified rHuTNF was labeled with ^{125}I using the IODO-GEN procedure as follows. Ten μg of rHuTNF in 40 μl of 1 M potassium phosphate buffer, pH 7.0, were layered over a freshly prepared film of IODO-GEN (10 μg) and incubated for 10 min at 4°C in the presence of 1 mCi of carrier-free $Na^{125}I$. The reaction mixture was brought up to 0.5 ml volume with PBS containing 0.1% gelatin, and the unreacted iodine was removed by gel filtration on a Sephadex G-25 PD10 column equilibrated with PBS containing 0.1% gelatin. The column was washed with 2 ml of the same buffer, and the flow-through volume was discarded. Radioiodinated TNF was eluted with the next 1.2 ml of the buffer. More than 95% of ^{125}I was incorporated into the protein as determined by trichloroacetic acid precipitation of total radioactivity and sodium dodecyl sulfate-PAGE in which a single band of TNF at M_r 17,000 was detected as radiolabeled. The specific radioactivity of the product was ~55 μCi/μg TNF.

Preparation of Acylated TNF. Acylated TNF was prepared using rHuTNF and the *N*-hydroxysuccinimide esters of fatty acids. The esters were synthesized by the method of Lapidot *et al.* (13). The reaction medium contained, in a final volume of 100 μl, 0.1 M sodium bicarbonate, various concentrations of the active ester of a particular fatty acid, 20 μg of cold rHuTNF, and 2×10^5 cpm of ^{125}I-rHuTNF. The reaction was started by adding various concentrations of the active ester dissolved in 10 μl of dimethyl-sulfoxide. After incubation at room temperature for 3 h with gentle stirring, the reaction mixture was applied to a Sephadex G-25 column (0.7 × 5 cm) previously equilibrated with PBS to remove residual active esters, *N*-hydroxysuccinimide, and dimethyl sulfoxide. Acylated TNF was fractionated by elution with the same

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[3] The abbreviations used are: rHuTNF, recombinant human tumor necrosis factor; DPPC, dipalmitoylphosphatidylcholine; PAGE, polyacrylamide gel electrophoresis; SUV, small unilamellar vesicle; PBS, phosphate-buffered saline; LD_{50}, 50% lethal dose.

buffer, and the radioactive fraction was used as the acylated TNF preparation.

Quantitation of Modified Residues. The extent of modification was determined by measuring the mobility of the modified proteins on native PAGE. Twenty μl of the acylation reaction mixture were applied to the lanes of a 4–12% linear gradient polyacrylamide gel, pH 8.29, and subjected to native PAGE as described (14). After silver staining, each protein band was individually cut from the gel, and the radioactivity was measured with a gamma counter. A weighted average of modified residues for each preparation was calculated from the counts in these protein bands.

Preparation of DPPC-SUVs. Ten mg of DPPC were first dissolved in a small volume of chloroform, and the solvent was dried under vacuum in a glass test tube. After addition of 1 ml of PBS (pH 7.4), the dried DPPC was hydrated with repeated vortex mixing at 50°C for 30 min. The suspension was sonicated at 50°C for 30 min using a probe-type sonicator (Model XL2020; Heat Systems, Farmingdale, NY) and then centrifuged at 1500 × *g* for 10 min to remove titanium particles.

Determination of Binding of TNF to DPPC-Vesicles. Binding of TNF to DPPC vesicles was determined by gel filtration as previously described (15). ^{125}I-native or ^{125}I-acylated TNF (5 μg; 5 × 10^4 cpm) was mixed with 500 μg of DPPC-vesicles and incubated at 20°C for 15 min. The incubation mixture was applied to a Sephadex G-200 column (1 × 15 cm) previously equilibrated with PBS, and vesicle-bound and unbound proteins were fractionated by elution with the same buffer. The radioactivity in each fraction (0.5-ml portions) was measured by gamma counting.

TNF Assay. TNF cytolytic activity was determined as previously described (16). L929 cells were plated at 15 × 10^3 cells/150 μl of Dulbecco-Vogt modified Eagle's minimum essential/F12 medium in 0.33-cm^2 wells. After overnight incubation, 50 μl of actinomycin D-containing medium were added to give a final drug concentration of 1 μg/ml. Samples of native or acylated TNF or their liposomal formulations were immediately titered on these targets, and incubations were continued overnight.

After 18–24 h, 50 μl of neutral red solution were added, and the remaining viable targets were allowed to incorporate dye for 60 min. Unincorporated dye was removed by washing once with PBS.

Incorporated dye was solubilized with acidified ethanol, and the A_{540} for each well was determined using a multichannel scanning apparatus. The LD_{50} of each sample was obtained from the titration curve of A_{540} and the TNF dose, and the relative cytolytic activity (%) was calculated from the reciprocal of the LD_{50} of the sample divided by that of native TNF.

RESULTS

Acylation of TNF with Long-Chain Fatty Acids. In our previous report, we showed that amino residues in rHuTNF are important for the expression of its biological activity, and the extent of modification (acetylation) of amino residues of TNF closely paralleled the extent of loss of cytolytic activity.[4] However, we also determined that low levels of modification (1–3 residues/trimer) can occur without significant loss of activity. Therefore, we attempted to acylate amino residues of TNF to low levels with long-chain fatty acids (C_8–C_{18}) to synthesize the lipophilic TNF.

Fig. 1 shows the effect of fatty acid chain length on the extent of acylation of TNF amino residues by the respective activated stress. In this case, TNF was modified by using a limited amount (5- and 10-fold molar excess) of active esters of each fatty acid to obtain a low level of modification.

Approximately equivalent extents of modification occurred with fatty acids from C_8 to C_{14} long, but the extent of modification decreased as the fatty acid chain length increased beyond this, and no significant modification was observed with C_{18}. Therefore, only acylated TNFs with fatty acid chain length from C_2 to C_{14} were used in the next series of experiments.

Fig. 2 shows the effect of fatty acid chain length and number of modified residues on the cytolytic activity of acylated TNF. In the case of acetylated TNF (C_2), modification at the level of 1–3 residues/trimer caused only a slight decrease (10–30%) in the cytolytic activity. On the other hand, the same level of modification with longer-chain fatty acids caused a more significant loss of activity. The extent of loss paralleled both the increase in the number of modified residues and the increase in the chain length of fatty acids. However, TNF coupled with 1–

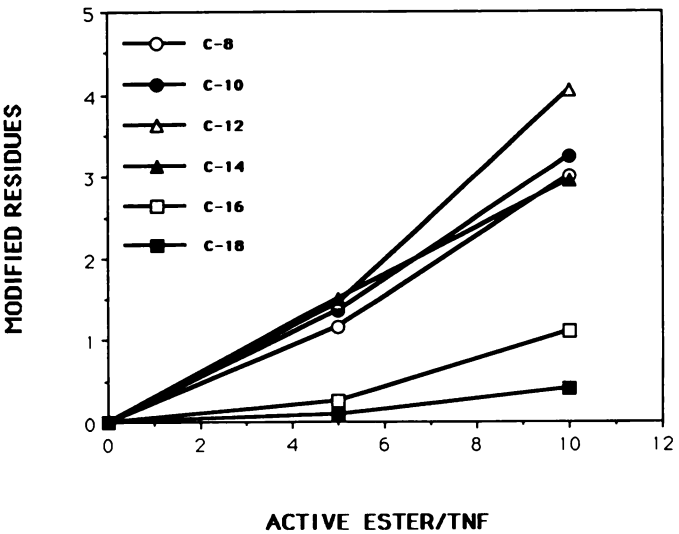

Fig. 1. Effect of mol ratio of active ester to TNF used for acylation and fatty acid chain length on extent of modification. ^{125}I-TNF was modified with 5- and 10-fold molar excess of active esters of each fatty acid (C_8–C_{18}), and the extent of modification was determined by measuring the mobility of the modified proteins on native PAGE. A weighted average of modified residues for each preparation was plotted against the molar ratio of active ester to TNF.

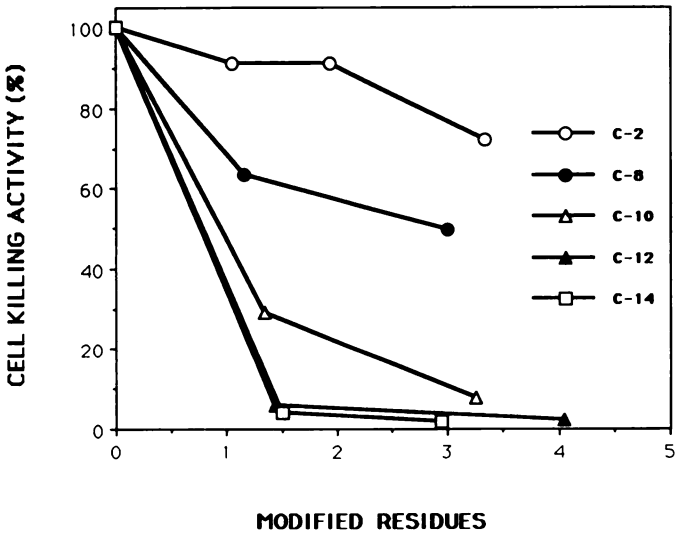

Fig. 2. Effect of extent of modification and fatty acid chain length on biological activity of TNF. Acylated TNF (C_8 ~ C_{14}) with different extents of modification was subjected to TNF assay on actinomycin D-treated L929 cells. The relative cytolytic activity (%) of each sample was calculated by comparing the reciprocal of the LD_{50} of the sample with that of native TNF and was plotted against the number of modified residues.

[4] T. Utsumi *et al.*, Molec. Immunol., in press

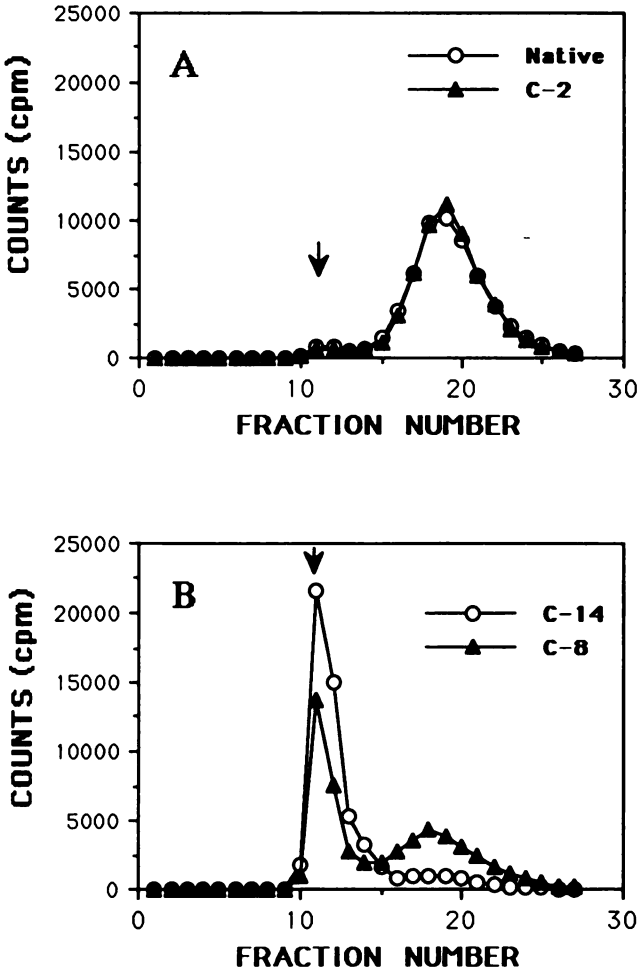

Fig. 3. Binding of ^{125}I-native, C_2-, C_8-, and C_{14}-TNF to DPPC-SUVs. Native ^{125}I-TNF or ^{125}I-acylated TNF (C_2, C_8, C_{14}) with an average modification of two residues was first incubated with DPPC vesicles at 20°C for 15 min and then subjected to Sephadex G-200 column chromatography. *Arrows*, position of DPPC-SUVs.

3.0 caprylic acid (C_8) chains/trimer retained ≥50% of the cytolytic activity.

Binding of Acylated TNF to SUVs. The affinity of acylated TNFs for lipid membranes was determined by binding of ^{125}I-native, ^{125}I-C_2-, ^{125}I-C_8-, and ^{125}I-C_{14}-TNF to DPPC-SUVs and quantitated by partitioning using Sephadex G-200 column chromatography. ^{125}I-TNF prepared as described previously was first incubated with DPPC-vesicles at 20°C for 15 min and then subjected to Sephadex G-200 column chromatography. Typical profiles of the separation of ^{125}I-TNF-DPPC-vesicle complexes from unbound protein are shown in Fig. 3. DPPC-vesicles or protein-DPPC-vesicle complexes were eluted in the void volume fraction (Fig. 3, *arrows*). As shown in Fig. 3*A*, binding of ^{125}I-native- or ^{125}I-C_2-TNF to DPPC-vesicles was negligible. In contrast, C_8-TNF, with an average modification of two residues effectively associated with vesicles as >50% of added protein, was detected in the vesicle fraction (Fig. 3*B*). In the case of C_{14}-TNF with the same level of modification, more than 85% of the added protein was found to be bound to the vesicles. Since these low-level acylations produced heterogeneous products, at least some of the unbound TNF is likely to be a nonacylated species.

Fig. 4 shows the effect of the number of modified residues on the binding of C_2- and C_8-TNF to DPPC-SUVs. C_2 acylation of TNF did not cause a detectable increase in the binding of TNF to DPPC vesicles, even when 6 residues/trimer were modified. In contrast, binding of C_8-TNF increased significantly as the number of modified residues increased; more than 80% of added TNF was bound to vesicles when the number of modified residues exceeded ~3.5 residues/trimer.

Cytolytic Activity of Liposome-associated C_8-TNF. To determine the cytolytic activity of liposome-associated acylated TNF, C_8-TNF bound to DPPC-vesicles was first purified by gel filtration to remove unbound proteins, and then the cytolytic activity of the complexes in the void volume was tested.

Fig. 5 showed the dose-response curves for the cytolytic activity of native, C_8- and DPPC-SUV-C_8-TNF. The C_8-TNF had an average of 2 caprylic acid chains/trimer. Free C_8-TNF

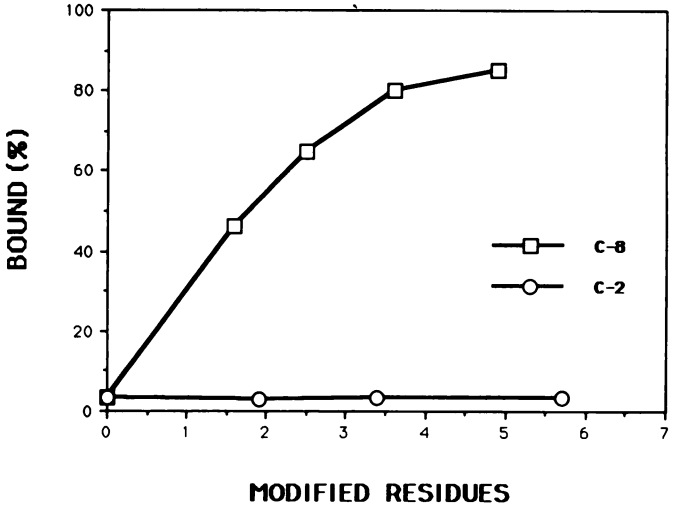

Fig. 4. Effect of extent of modification of TNF with C_2 or C_8 chains on binding to DPPC-SUVs. ^{125}I-C_2-TNF and ^{125}I-C_8-TNF with different extents of modification were subjected to the binding assay with DPPC SUVs as shown in Fig. 3. The extent of binding (%) was plotted against the number of modified residues. ○, ^{125}I-C_2-TNF; □, ^{125}I-C_8-TNF.

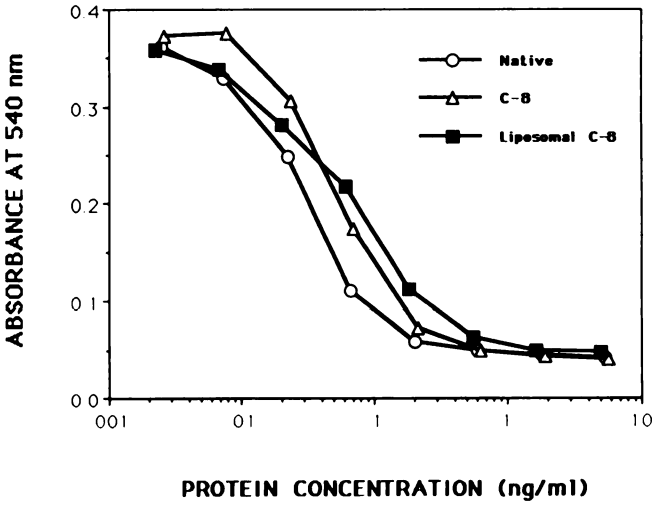

Fig. 5. Biological activity of native, C_8-, and DPPC-SUV-C_8-TNF. Dose-response curve for neutral red incorporation by L929 targets against protein concentration. Native TNF, C_8-TNF, and liposomal C_8-TNF bound to DPPC-vesicles were first purified by gel filtration to remove unbound proteins and then subjected to TNF assay on actinomycin D-treated L929 cells. C_8-TNF had an average of 2 caprylic acid chains/trimer.

shows slightly decreased cytolytic activity (~60% of native TNF), but otherwise the shape of its dose-response curve was quite similar to that of native TNF. DPPC-SUV-C_8-TNF demonstrated almost the same LD_{50} as free C_8-TNF; however, the profile of the dose-response curve was slightly different from that of C_8-TNF, showing relatively stronger activity in the low-protein-concentration range.

DISCUSSION

The purpose of this investigation was severalfold, to determine whether: (*a*) lipophilic adducts of rHuTNF could be produced by chemical synthesis; (*b*) they could exert biological (cytolytic) activity *in vitro*; (*c*) they would show high affinity for liposome membranes; and (*d*) the liposomal-lipophilic-TNF preparations retained biological activity. Our results clearly confirm all of these assumptions for DPPC-SUV-C_8-TNF.

The marked susceptibility of the biological activity of rHuTNF to the chemical introduction of hydrophobic fatty acid side chains, with respect to both number and length, was a significant constraint on the strategy used. As we have reported,[4] amino functions are required for biological activity of TNF, and only limited modification is well tolerated. Other studies have demonstrated that proteins with enhanced hydrophobicity due to acylation are well capable of interacting with other macromolecules; *e.g.*, lysozyme could be monoacylated with fatty acid chains from C_8 to C_{14}, thereby demonstrating affinity for phosphatidyl serine/DPPC vesicles, and was still effectively phosphorylated by bound protein kinase C (17). In contrast, acylation of TNF with long-chain fatty acids, even at low levels (1–2 residues/trimer), markedly perturbed cytolytic activity (Fig. 2). Direct competitive binding studies should determine whether this is due to reduced affinity for its receptor. Alternatively, other postbinding steps may be affected, although recent studies with a noncleavable form of the human prohormone indicate that cytolysis can occur in the absence of internalization of TNF (18). Thus, the SUVs displaying lipophilic TNF anchored on their outer surface may transduce their lytic signal via the TNF receptor. Given the previously described high affinity of myristylated proteins for lipid membranes, another possible explanation for inactivation is hydrophobic entrapment in the target cell membrane of the lipid-modified TNF so as to sequester it from productive and necessary interactions with the target cell TNF receptor. These mechanisms will be addressed in future studies.

The identity of the most readily modified residue(s) is also of particular interest. Of the six lysyl residues in the monomer, two, Lys 11 and Lys 98, are involved in intra- or intersubunit salt bridges (19). Of the remaining four, Lys 90 and Lys 128 are near the base of the trimer, and their modification could be expected to perturb receptor interactions (19). The lysines at positions 65 and 112 are near the top of the trimer and should be available for modification without such constraints. Some models of nucleophilic substitution reactions would predict that the α-amino group of the NH_2-terminal valine should be more reactive than the lysyl ϵ-amino groups. This hypothesis can be evaluated in the future by NH_2-terminal amino acid determinations of native and acylated TNFs. If this is the case, the high mobility of the NH_2 terminus (19) and the enhanced biological properties conferred by increasing its basicity (20–22) suggest additional approaches to developing potent, lipophilic TNF derivatives. For example, introduction of sequences encoding myristylation signals at the amino-terminus (23, 24) could obviate the need for further chemical acylation with its concomitant compromise of biological activity. Anchoring of the TNF to the liposome surface via its amino-terminus may be advantageous because this is akin to the orientation of the transmembrane prohormone on the effector cell surface (25).

Nevertheless, we have demonstrated that it is possible to prepare with high efficiency biologically active liposomal TNF starting from the parental molecule. It will be of considerable importance to compare its pharmacokinetic characteristics to those of the native TNF-multilamellar vesicle preparations previously described by Debs *et al.* (11, 12). A SUV preparation such as our formulation may be suitable for enhancing delivery of TNF to the tumor, due to its avoidance of rapid capture by the reticuloendothelial system and to the vectorial display of the toxin on the exterior liposomal surface.

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