Recombinant Human Tumor Necrosis Factor-β
Entrapped in Liposomes Formed by a Modification
of the Dehydration-Rehydration Method Retains Potent
Cytotoxic Activity on L929 Cells In Vitro

Lloyd Tan¹, Cynthia R. Goh² and Alan G. Porter²

¹Department of Pharmacology, Faculty of Medicine, National University of Singapore, Kent Ridge, Singapore 0511
²Institute of Molecular and Cell Biology, National University of Singapore, Kent Ridge, Singapore 0511

Received January 28, 1992 Accepted March 9, 1992

ABSTRACT — Recombinant human tumor necrosis factor-β (rhTNF-β) may be encapsulated with high
efficiency in phosphatidylcholine and distearoylphosphatidylcholine liposomes, with entrapment values
of 93.4% and 92.3%, respectively, by first entrapping the substance in multilamellar vesicles using a
high solute-to-phospholipid ratio followed by freeze-drying and then rehydration. The entrapped cyto-
kine retains potent cytotoxic activity on L929 cells in vitro, causing 100% cytotoxicity, equal to that of
free rhTNF-β at a concentration of about 5 × 10⁻⁸ g/ml.

Keywords: Liposomes, Tumor necrosis factor-β, L929 cells

Tumor necrosis factor-β (TNF-β), or lymphotoxin, is
a 20–25-kDa glycosylated protein which is 169–171
amino acid residues long (1). It was discovered as a
substance produced by activated T lymphocytes that
was associated with the 24-hr inflammatory response
known as delayed hypersensitivity. TNF-β is 28% iden-
tical and about 52% homologous, at the amino acid
level, with tumor necrosis factor-α (TNF-α) and is also
able to cause dramatic necrosis of tumors. The success-
ful cloning of human TNF-β (2, 3) as a non-glycosylated
but fully active recombinant protein (rhTNF-β) in E.
coli has made it more widely available as a laboratory
reagent and facilitated further elucidation of its biologi-
cal properties.

Liposomes are vesicles consisting of bilayers of
amphiphilic molecules such as phospholipids (4). They
are extremely versatile structures and can be formu-
lated using phospholipids of varying gel-to-liquid crystal
transition temperatures to arrive at vesicles of different
sizes, lamellar characteristics and pharmacokinetic and
pharmacodynamic properties. They can also be made to
contain almost any conceivable substance up to the size
of viral particles.

It would be interesting to determine if rhTNF-β en-
capsulated in liposomes would retain the bioactivity of
the native lymphokine because a liposomal formulation
of rhTNF-β could theoretically possess several advan-
tages in the treatment of patients with cancer, for ex-
ample, a reduction in the incidence of systemic side
effects. In this paper, we describe a modification of the
dehydration-rehydration method which results in much
higher encapsulation efficiencies, as well as demonstrate
that liposome-encapsulated rhTNF-β retains potent
cytotoxic activity on mouse L929 cells in vitro.

rhTNF-β was prepared according to Seow et al. (3).
Egg phosphatidylcholine (PC) or distearoylphospha-
tidylcholine (DSPC) (50 μmoles) and 50 μmoles
cholesterol (Chol) (Sigma) were mixed in a 50-ml
round-bottom flask (Quickfit). The lipids were dried to
a thin film by evaporation of the solvent at a low speed
in a rotary evaporator (Buchi) connected to a running
tap-water pump. Intermittent insufflations of oxygen-
free nitrogen were let into the flask to expedite the pro-
cess. One milliliter of phosphate-buffered saline (PBS,
pH 7.4) containing rhTNF-β at a concentration of 5
μg/ml (with ¹²⁵I-labelled tracer) was added to the dried
lipid film. The flask was lowered into the water-filled
cup of a cell sonicator (Sonic & Materials Inc.,
U.S.A.) and subjected to bursts of sonication while
being manually rotated to form multilamellar vesicles
(MLV). The preparation was lyophilized overnight fol-
lowed by two-step rehydration with 0.1 ml distilled wa-

ter and 0.9 ml PBS. rhTNF-β entrapment was estimated by assaying 125I radioactivity in the pellet after washing three times in 8 ml PBS by centrifugation at 10,000 × g for 30 min.

To assay the formulations for rhTNF-β for cytotoxicity, 5 × 10^4 L929 cells cultured in RPMI 1640 containing 10% fetal calf serum (FCS) were plated on in each well (×96) and grown overnight at 37°C. After removal of the medium, 100 μl of serial dilutions of rhTNF-β in test medium were then pipetted into the appropriate wells in quadruplicates to give final concentrations of 1% FCS and 10 μg/ml cycloheximide (Cx). Untreated (control) wells contained only 100 μl of test medium. After 18 hr of incubation at 37°C, the monolayers were washed in PBS, stained (0.5% crystal violet in 20% methanol) and then washed again. The stain was eluted into 100 μl of 33% acetic acid and the absorbance at 550 nm (A550) quantitated with a microwell plate reader. The percentage cytotoxicity was defined as:

\[ \frac{(A550 \text{ in test well}) - (A550 \text{ in untreated well})}{(A550 \text{ in untreated well})} \times 100 \]

Using a modification of the method for the formulation of dehydration-rehydration vesicles (DRV) as described by Kirby and Gregoriadis (5), high and reproducible percentage entrapment values could be obtained with both PC as well as DSPC DRV (Table 1). We found that 93.4% and 92.3% of the total of 5 μg rhTNF-β were entrapped in PC and DSPC DRV, comprised of 50 μmol phospholipid and equimolar cholesterol, respectively. It was found that the intermediate stage before the lyophilization of a mixture of small unilamellar vesicles (SUV) and the substance to be entrapped, namely the probe-sonication of MLV to form SUV, can be eliminated entirely if the substance to be entrapped is first encapsulated in MLV using the method originally described by Bangham (6). This places the substance in more intimate contact with the phospholipid bilayers prior to freeze-drying. Additionally, the highest amount of phospholipid or the smallest amount of a solution of the substance that will enable the formation of liposomes was used. The remaining unentrapped substance is then lyophilized together with the MLVs. On rehydration, more than 90% of the original substance added may be entrapped. Obvious advantages of eliminating the MLV-to-SUV conversion step are the shorter time taken for the whole process of DRV liposome formulation, the smaller amounts of unencapsulated substance wasted, the facility of scaling up this simplified process, the prevention of potential oxidation of phospholipid and cholesterol by the sonication process, the elimination of the steps required for the removal of titanium probe particles liberated into the SUV preparation and for the rejection of malformed SUV preparations that are not sufficiently clear.

Kirby and Gregoriadis (5) in first reporting the DRV method found that leaving out the MLV-to-SUV conversion step and merely lyophilizing MLVs with the substance to be encapsulated yielded entrapment values that were still high compared to Bangham's original method (6). However, these fall short of values obtained when a solution of the substance was freeze-dried with SUVs. One may hypothesize that the increased entrapment is due to the greater surface area of the SUVs available to be in close apposition to the substance facilitating their inclusion into the DRV bilayers during the rehydration stage. Lyophilized MLVs are not as efficient as many of their bilayers are internal to the outermost layer and thus not in intimate contact with the substance to be entrapped. However, using the modifications described, this deficiency was circumvented by incorporating the substance within the internal lamellae of the MLVs prior to lyophilization. The high encapsulation efficiencies obtained lend support to the hypothesis. There were no marked differences in encapsulation values using two phospholipids of widely differing gel-to-liquid crystal transition temperatures.

Another technique found to expedite the process of deposition of phospholipid and cholesterol in a thin layer at the bottom of the round-bottom flask was to allow whiffs of nitrogen gas from a nitrogen cylinder connected to the inlet, which normally merely allows outside air into the low-pressure environment of the flask at the end of the procedure, into the flask at one to two-minute intervals during the rotary evaporation process itself. Care must be taken to ensure that the sudden inrush of nitrogen does not sufficiently raise the pressure inside the flask to above atmospheric pressure.

| Liposome composition | PC : Cholesterol | DSPC : Cholesterol |
|----------------------|------------------|--------------------|
| % Entrapped          | 93.4 ± 1.2 (4)   | 92.3 ± 0.8 (4)     |
The cytotoxic effect of rhTNF-β either free or encapsulated in DRV liposomes on L929 cells was determined using a vital dye to stain the remaining live cells after incubation for 18 hr. It is seen (Fig. 1) that at a rhTNF-β concentration of about 5 \times 10^{-8} \text{g/ml}, all the cells are killed by free as well as liposome-encapsulated rhTNF-β when the translation inhibitor, cycloheximide is present in the incubation medium. When cycloheximide is absent, cytotoxicity is markedly reduced with free rhTNF-β causing about 40%, rhTNF-β encapsulated in PC DRV, 32% and rhTNF-β in DSPC DRV, 18% cytotoxicity. Without cycloheximide, rhTNF-β cytotoxicity falls to zero at concentrations of around 5 \times 10^{-10} to 5 \times 10^{-11} \text{mg/ml}. However, in the presence of cycloheximide, all formulations of rhTNF-β retain strong cytotoxic activity even at very low concentrations. Thus, at femtogram concentrations and below, the formulations are still more than 50% cytotoxic on L929 cells, with free rhTNF-β being more efficacious than that entrapped in PC DRV, which in turn appears to be more cytotoxic than the DSPC DRV formulation. A possible reason for this could be that after internalization of the liposomes by the cells, the rhTNF-β buried more deeply in the internal layers of the multilamellar liposomes cannot escape into the cytoplasm within the short period of incubation so that a large proportion, although intracellular, remains dormant unlike the case with the unentrapped substance where all intracellular molecules are free to exert their effects. This postulation is supported by the fact that the more rigid liposomes composed of DSPC, with a higher gel-to-liquid crystal transition temperature, are less cytotoxic than PC DRV which are more fluid at the incubation temperature. rhTNF-β molecules in the interior of

Fig. 1. Comparison of the cytotoxicity of free and liposomal recombinant human tumor necrosis factor-β (rhTNF-β) on L929 cells in vitro. Formulations of rhTNF-β, either free or encapsulated in phosphatidylcholine (PC) or distearoylphosphatidylcholine (DSPC) liposomes, were incubated for 18 hr with L929 cell monolayers in medium containing cycloheximide (Cx) or in medium without Cx. The percentage of cells killed was determined by staining the remaining live cells with a vital dye, quantitated with a microwell plate reader, and comparing the values with those of stained untreated cells (see text for details). Values shown are the means of four readings \( \pm \) one standard deviation. ■: Free TNF-β (with Cx), ◇: TNF-β in DSPC (with Cx), ▲: TNF-β in PC (with Cx), ●: Free TNF-β, ▼: TNF-β in DSPC, ◆: TNF-β in PC.
DSPC DRV may be more shielded against perhaps lysosomal enzymes that would liberate them into the cytoplasm.

It is likely that rhTNF-β entrapped in liposomes does not need to be bound to the TNF receptor located at the cell membrane to gain entry into the cell. This would be advantageous if an antitumour effect is desired in a cell line or an in vivo tumor that did not express the TNF receptor. Previous studies (7) have shown that liposomes may gain entry into a cell by endocytosis, by fusion of the liposomal lamellae with the cell membrane or by cell-induced lysis of the liposomes, thus liberating their contents into the cell. These would probably also be the main mechanisms operative here. The ways in which TNF-β cause cytostasis and cytolysis are complex and incompletely understood (8). One postulation is that after internalization, it damages lysosomes, and normally sequestered lysosomal proteins such as endonucleases are liberated which could cause DNA damage and cell death. Receptor binding may only be for the purpose of internalization as neither the number of receptors per cell nor the binding affinity correlates with the susceptibility of a cell line to cytoly sis. Tachyphylaxis or resistance to the cytolytic effects of TNF-β in several cell lines is dependent upon the synthesis of 'resistance proteins' (3). That is why exposure of cells to the protein translation inhibitor cycloheximide markedly reduces the time taken for maximal cytolytic effects to be seen and accounts for the difference in rhTNF-β cytotoxicity on L929 cells in the presence and absence of cycloheximide seen in this experiment.

A recent study (9) using TNF-α entrapped in multilamellar liposomes revealed that 1000 U/ml TNF-α either surface-adsorbed or internally entrapped were equally efficacious in causing lysis of WEHI-164 murine fibrosarcoma cells when compared with free TNF-α. Our data using TNF-β are perfectly in keeping with these results. Thus, both TNF-α and rhTNF-β may be administered in liposomes without much diminution in cytotoxic effects. Liposomal formulations containing TNF may indeed be more advantageous in the treatment of liver tumors which do not express the TNF receptor as the liposomes would ‘target’ them to the liver where they would gain entry into the tumor cells without the need to first bind to the receptor. The systemic adverse effects of large amounts of TNF would also be lessened. Moreover, liposome-encapsulation avoids the cytotoxicity-neutralizing effect that anti-TNF-α antibodies have on free TNF-α.

In conclusion, we have shown that rhTNF-β may be encapsulated with high efficiency by first entrapping the substance in MLV using a high proportion of phospholipid followed by freeze-drying and rehydration. The entrapped cytokine retains potent cytotoxic activity on L929 cells in vitro.

Acknowledgment
This study was supported by National University of Singapore grant RP 890262

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