Delivery of Soluble Tumor Necrosis Factor Receptor from In-Situ Forming PLGA Implants: *In-Vivo*

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INTRODUCTION

Many harmful effects of tumor necrosis factor (TNF) result from chronic formation of this cytokine at certain distinct loci in the body. The soluble forms of the TNF receptors p55 and p75, which have the ability to block TNF action by competing for it with the cell surface TNF receptors (1–4), can protect against harmful pathological effects of TNF in chronic inflammatory diseases. Chronic inflammatory diseases are associated with increased endogenous formation of the soluble TNF receptors, in part resulting from stimulation of their synthesis by TNF (5–12). These receptors are formed by proteolytic processing of the cell surface TNF receptors and correspond to the cysteine-rich ligand-binding regions in their extracellular domains.

Since sp55-R are cleared rather rapidly from the blood, repeated injections may not maintain them at the required site, at high enough concentrations. Due to the short half-life (6 hours) of this protein in-vivo, a controlled delivery system based on implants of ethylene vinyl-acetate (EVAc) or injections of microspheres of poly (lactic-co-glycolic) acid (PLGA) copolymers containing human soluble p55 TNF receptors (13), could enhance the therapeutic potential of the natural soluble TNF receptors in chronic diseases.

The objective of this study was to develop a drug delivery system with the benefits of an implant but the ease of administration of an injection to the site of need. This delivery system involves simple preparation procedures and avoids an invasive technique such as surgery in its implantation or removal. We have developed an in-situ forming implant system that can be administered as a liquid using standard syringes and needles. A biodegradable injectable delivery system was described recently by Dunn et al. (14). Upon contact with body fluids, the liquid system coagulates to form a solid implant for drug delivery. We looked for materials that have been approved for human application. We present a system comprised of a bioactive agent and a water insoluble biodegradable polymer (poly lactic-co-glycolic acid, PLGA) dissolved in a pharmaceutically acceptable water-miscible solvent (glycofurol). PLGA is a commercially available product approved by the FDA for human application. Glycofurol has been used as a solvent in parenteral products for intravenous, intranasal or intramuscular injection (15–21).

We show that the injectable in-situ-forming controlled release system used in-vitro (22), is able to increase the soluble TNF receptor serum concentrations such as occurs in chronic inflammatory diseases in a controlled manner in-vivo. Furthermore, we demonstrate that this system can be used as a long-term protection devise against the harmful pathological effects of TNF.

MATERIALS AND METHODS

Preparation of PLGA Formulations

The copolymer PLGA 75:25 (Boheringer, Resomer RG 755, i.v. 0.59 dl/g) was dissolved in glycofurol (α-[(tetrahydro-2-furanyl) methyl]-w-hydroxy-poly(oxy-1,2-ethanediyl); Sigma Chemical Co., USA) at room temperature. The proteins were incorporated into the PLGA solution in a dispersed form. Powders of sp55-R (the human recombinant protein, Mw = 36 kD, produced in Chinese hamster ovary cells (CHO), InterPharm Laboratories Ltd., Israel), BSA (Sigma Chemical Co., USA) or a mixture of sp55-R and BSA (sp55-R/BSA solution containing sp55-R, BSA and 0.5 mM sodium phosphate buffer, pH 6.0, was freeze dried using a Labconco Lyph lock-6 freeze-drier) were sieved, yielding particles of 75–100 μm. The protein particles were dispersed into a 3 ml PLGA solution in a 15 ml tube using vortex (Press-to-mix, 34524, Snijders) at maximum speed for 3 seconds and probe sonication (Model VC-250, Sonics and Material Inc.) at output 4 (50 W) for 30 seconds, while the solution was kept on ice.

Determination of the Release Characteristics

In Vitro

In vitro release profiles were obtained by injecting 350 μl of the suspensions through a needle directly into 10 ml of phosphate buffered saline (PBS, pH 7.4) contained in a vial (20 ml capacity) to form a solid device. The study was carried out at 37°C in a shaker bath. Samples of the release media were withdrawn periodically and BSA release was quantified by absorbance at 280 nm using a spectrophotometer (Spectronic Model 1201, Milton Roy Ltd.). sp55-R levels were determined by a 2-site capture Enzyme Linked Immuno Sorbent Assay (23). After every sampling the PBS solution was decanted from the solid device and replaced with fresh PBS. The amount of protein in PLGA implants reflect the actual percentage of protein entrapped in the system allowed to age in PBS as determined by two methods: directly by recovering the protein from PLGA implants and indirectly by measuring the residual unentrapped protein in the outer water phase.

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ABBREVIATIONS: TNF, Tumor necrosis factor; sp55-R, soluble p55 TNF receptors; EVAc, ethylene vinyl-acetate; PLGA, poly (lactic-co-glycolic) acid.

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In Vivo

The PLGA-protein solutions were injected into mice subcutaneously, in the abdominal area. Comparison of the serum concentrations of human sp55-R in mice injected with the protein, to the serum concentrations in mice injected with polymeric formulations was done using Balb/c and Balb/c nude female mice, aged 8–9 weeks (weighting 20–21 gr).

Effects of the injectable polymeric systems were examined in experimental mice model for chronic inflammatory diseases. The model used was for the cachectic effect of TNF. As reported previously by Oliff et al. (24), nude mice bearing TNF-producing tumors exhibit severe cachexia, leading to death. Balb/c nude mice (aged 8–9 weeks and weighing 20–21 gr) were inoculated subcutaneously in the flank area with 0.1 ml PBS containing 1×10^7 freshly trypsinized CHO cells expressing the human TNF gene (CHO/TNF cells (25)). Injections of the PLGA formulations to these mice were performed 5 days after tumor cell inoculation. In two of the controlled groups, 100 μg/mouse of mouse monoclonal anti-TNF antibodies (TNF-1, established in the laboratory of D.W.) were injected intraperitoneally, once (5 days after tumor cell inoculation) or twice with a time gap of one week (days 6 and 13).

Serum samples were collected periodically after injections by tail bleeding and via an eye artery, and allowed to clot; their sp55-R levels were determined by a 2-site capture Enzyme Linked Immuno Sorbent Assay (23).

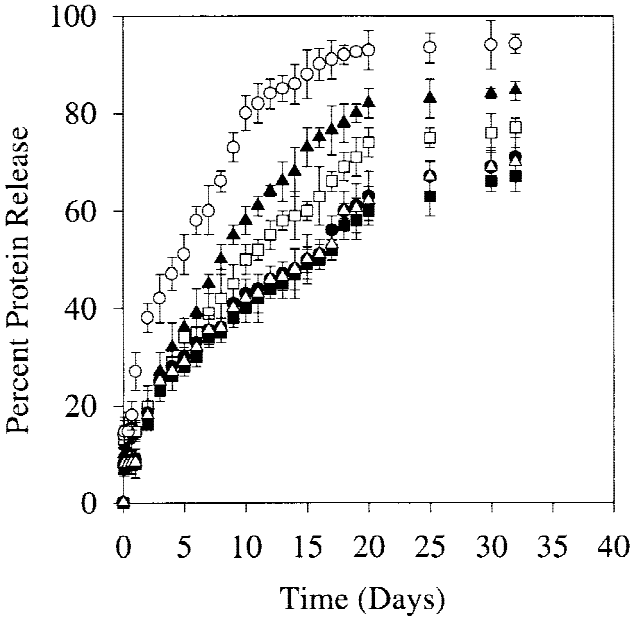

Fig. 1. Effect of BSA on the release kinetics of sp55-R from PLGA 75:25 devices. Formulation contained 10% polymer in glycofurol by weight at total protein loading of 10% wt. protein/wt. polymer [BSA (○), a mixture of BSA and sp55-R at weight ratio of 20:1 respectively (▲), or sp55-R (□)] or 3% wt. protein/wt. polymer [BSA (●), a mixture of BSA and sp55-R at weight ratio of 20:1 respectively (△), or sp55-R (■)]. Each point represents the mean of seven samples, presented with standard deviation bars.

RESULTS AND DISCUSSION

In order to obtain a continuous and constant release of the soluble receptors in-vivo, we designed an in situ forming biodegradable polymeric delivery system based on PLGA that contains the human p55 TNF receptor (sp55-R), optimizing the weight percent of the polymer in solution, ratio of polylactide to polyglycolide and protein loading level in the polymer, for maximal rate and duration of soluble receptor release.

sp55-R is a highly potent receptor, exerting its biological effects on a nanogram scale. To increase the entrapped protein mass, bovine serum albumin (BSA), an inert protein that has no effect on the function of neither TNF nor sp55-R, was co-entrapped with the soluble receptors (22,26). Figure 1 shows the release profiles of BSA and sp55-R from PLGA injectable implants, in-vitro (22). The formulation, composed of PLGA 75:25 containing a mixture of BSA and sp55-R (20:1 weight ratio) at a total protein loading of 3% or 10% (wt. protein/wt. polymer), exhibited protein burst within the first day of incubation, followed by a continuous release of the proteins over the next 20 days. As can be seen the release of proteins from these implants even at the low loading concentration of 3% is fast in comparison to the degradation of the PLGA 75:25 copolymer that has a degradation half-life of months. We therefore propose the protein release from these implants is controlled by combination of matrix degradation, diffusion and an osmotic-mediated mechanism induced by residual entrapped glycofurol. It appears that at low loading level the osmotic mechanism is the major contributor. At higher loading levels, BSA acts as a carrier mediating the continuous release of sp55-R and facilitates the diffusion of sp55-R by increasing the number of pores in the polymeric matrix (22). Recently, we showed (22) that variations of the polymer composition, molecular weight, and concentration could be used to provide formulations with the desired release rates and duration of release. Moreover, the biological activity and stability of the soluble receptors were not lost during the processing of these devices (22). The next step was to assess the function of these controlled release systems in vivo.

Figure 2 shows the sp55-R serum concentration level of nude mice injected subcutaneously with polymeric formulations containing a mixture of BSA and sp55-R at two different loading levels of 3 and 10% total protein (wt. protein/wt. polymer; the concentration of the polymer in glycofurol was 10%). Similar to the in-vitro results presented in Figure 1, both loading levels exhibited almost the same level of protein burst within the first day of incubation, followed by more stable profile of sp55-R serum concentrations. At 10% loading level, sp55-R serum concentration remained constant at a 30–35 ng/ml for 35 days. However, at 3% loading level, sp55-R serum concentration remained constant only for a period of 20 days at a 20–25 ng/ml, and then declined until total depletion after 28 days.

Subcutaneous injection of polymeric formulations at two polymer concentrations containing sp55-R into nude mice resulted in constant elevated levels of sp55-R for more than 3 weeks (Figure 3A). Following the initial burst, sp55-R serum concentration at 6 hours after the formulation injection is similar for both of the polymer concentrations 10 and 20%. These results commensurate with the in-vitro results (Figure 3B) which indicated that following the initial burst, there is no significant effect of the different polymer contents in the injecting formulations on protein release rates.

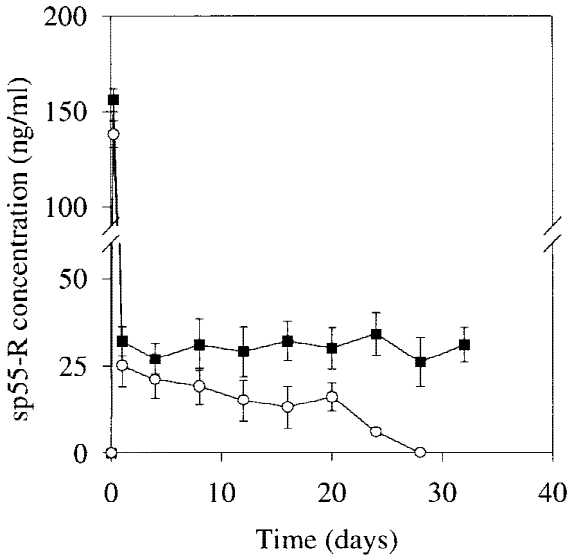

Fig. 2. Effect of loading level of protein on sp55-R serum concentration. 0.05 gr PLGA 75:25 formulations loaded with 3% (○) or 10% (■) sp55-R together with BSA (wt. protein/wt. polymer; 7.1 or 23.8 μg sp55-R, respectively) were injected subcutaneously into Balb/c nude mice. BSA/sp55-R weight ratio was 20:1. Polymer concentration in glycofurol was 10% (wt. PLGA/wt. glycofurol). Each point represents the mean of the data in group of eight mice, presented with standard deviation bars.

The release profiles of all studied systems are characterized by a typical initial protein burst followed by constant release rates (Figures 1–3). We propose the protein burst is due to protein release from the matrices during solidification. Since injectable implants are administrated as a liquid, it is reasonable to assume that during the lag time between the injection and the formation of the solid implant, the proteins diffuse out causing the burst effect (22). sp55-R serum concentrations at 6 hours after injection are similar for the two formulations with polymer concentration of 10 and 20% as shown in Figure 3A. When the implants solidify, glycofurol escapes from the polymer solution, facilitates the migration of proteins onto the surface of the implants. It appears that a high polymer concentration (higher viscosity of the injected solution) limits the diffusion of proteins out of the suspension during the lag time between the injection and the formation of the solid implant (i.e. limits the burst effect), as evidenced by the decrease in initial burst as polymer concentration rises. In addition, for higher polymer concentration the solution solidifies faster trapping more proteins in the implant (reaches faster the solubility limit, when glycofurol escapes from the solution). However, the long-term release kinetics after the burst were independent of polymer concentration in solution as can be seen in Figure 3B.

The solidifying rate of the polymer is crucial to preserve the controlled release function following the burst, since slower precipitation might result in a burst of all entrapped proteins. It can be seen that the initial burst in-vivo is slightly higher than in-vitro. Since the system was injected subcutaneously, decrease of the burst could be achieved by increasing the speed of injection, which will result in formation of a more compact structure of the implant. Both the in-vitro and in-vivo data suggest that the solidifying rate of the polymer can

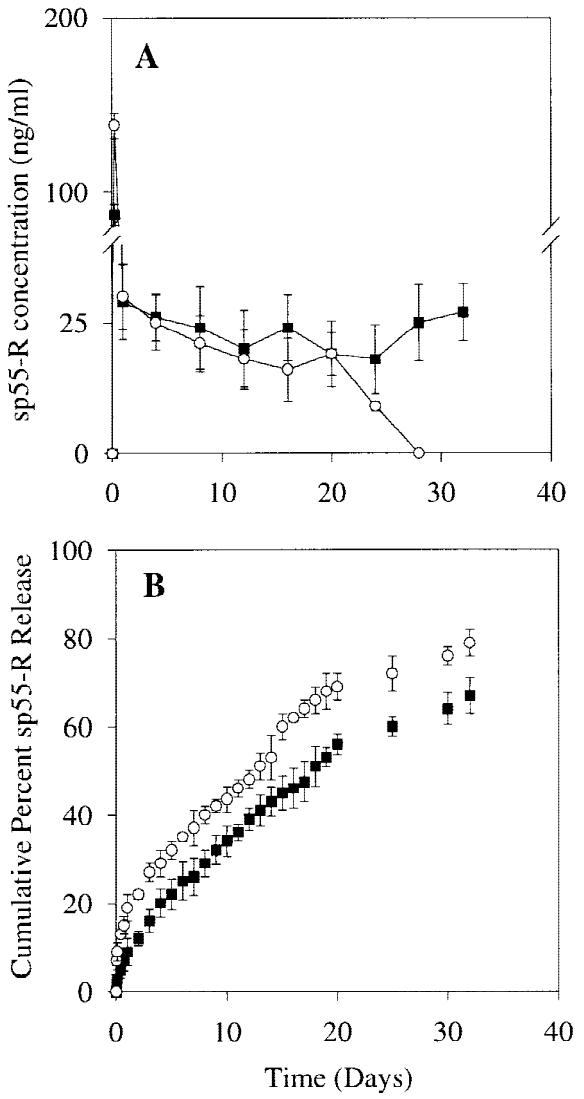

Fig. 3. Effect of polymer concentration in glycofurol on sp55-R release. (A) Mice serum concentration of sp55-R and (B) the release kinetics in-vitro of sp55-R from PLGA formulations following injections of 0.05 gr formulations polymer (10% wt. PLGA/wt. glycofurol, sp55-R together with BSA (7.1 μg sp55-R) (○) or 20% wt. PLGA/wt. glycofurol, sp55-R together with BSA (14.2 μg sp55-R) (■). BSA/sp55-R weight ratio was 20:1. Proteins loading level was 3% (wt. protein/wt. polymer). Each point represents the mean of the data in group of eight mice (A) or samples (B), presented with standard deviation bars.

be also controlled by polymer concentration in the administered solution. It is important to note that animals show no visible signs of discomfort or overt toxicity while the implant solidified.

To evaluate the utility of the release of the sp55-R from the polymeric implants as a means of protection against the pathological effects of chronic exposure to TNF, we examined the effect of injected implants in an experimental mice model (Figure 4). The model used was for the cachectic effect of TNF. As reported previously (24), nude mice bearing TNF-producing tumors exhibit severe cachexia, leading to death. Injection of PLGA formulations containing sp55-R into the tumor-bearing mice (about 7 μg of the receptors per mouse)

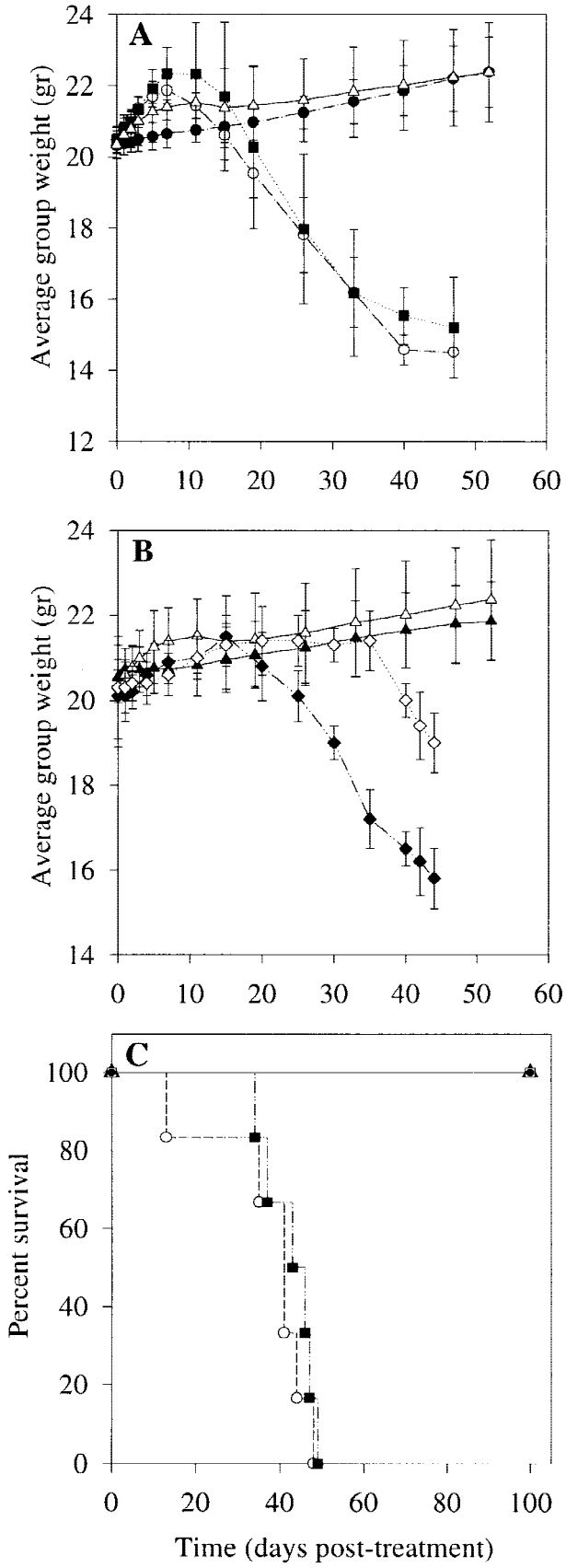

Fig. 4. Effect of sp55-R (A) and of an anti-TNF antibody (B) on the weight, and of the sp55-R on survival (C) of mice that bear TNF-producing tumors by injection of polymeric formulation that release sp55-R. (A, B) Weight and (C) Survival of: healthy Balb/c nude mice (●); healthy Balb/c nude mice injected with empty polymeric formulation (10% wt./wt. polymer in glycofurol) (□); Balb/c nude mice that bear a TNF-producing tumor (CHO/TNF), without any treatment (○); injected with 0.05 gr empty polymeric formulation (10% wt./wt. polymer in glycofurol) (■); injected with 0.05 gr polymeric formulation (10% wt./wt. polymer in glycofurol, 3% loading of sp55-R together with BSA [7.1 μg sp55-R]) (△); injected with 0.05 gr polymeric formulation (20% wt./wt. polymer in glycofurol, 3% loading of sp55-R together with BSA [14.2 μg sp55-R]) (▲); injected with neutralizing monoclonal anti-TNF antibody (100 μg/mouse), once (♦) or twice with a time gap of one week (◇). BSA/sp55-R weight ratio was 20:1. Each point represents the mean of the data in group of eight mice, presented with standard deviation bars.

prevented body weight loss (body weight increased naturally) and mortality was prevented for the duration of the experiment (Figure 4C). No protection was observed when injecting control polymers, lacking sp55-R (Figure 4A), while the protective effect of injected anti-TNF antibody persisted for only a few days (Figure 4B). Even mice injected twice with the antibody eventually developed progressive wasting and died. The results in Figure 4 suggested that the biological activity of the soluble receptors was not lost during the processing of this delivery approach. Injections of the novel injectable polymeric formulations containing the sp55-R into tumor-bearing mice prevented body weight loss and mortality. As a result the weight of mice treated with the injectable implants containing sp55-R increased as in healthy mice.

Additional aspect of the developed system is the fact that when formulated, it maintains a liquid consistency that allows injection through a needle. The needle diameter is of great importance, since the thinner the needle the more tolerable the injection will be, leading to greater acceptance by patients. The formulations can be injected via needles in the range of 19-22 gauge depending on the concentration and the molecular weight of the polymer. These needles are the most popular needles in use for intramuscular injections of drugs to humans.

The novel approach presented here demonstrates a delivery method for the natural receptors that would successfully reproduce the natural phenomenon. Three major features of mode of delivery were achieved with this system: (1) Small amounts of the soluble receptors (far less than needed for bolus injection) suffice to maintain prolonged and significant elevation of their concentration in serum. (2) By modulating key formulation variables like protein loading level and polymer concentrations, the desired release rate and burst effect can be achieved. (3) This sustained release system provides effective protection against pathological manifestations of chronic exposure to TNF. Since the sp55-R is of human origin, the delivery of the natural receptors in human would reproduce more faithfully the way it occurs in nature.

CONCLUSIONS

This work presents a novel approach for sp55-R delivery system. In this study, we have demonstrated the efficacy of the injectable polymeric controlled release systems in a rodent model for chronic action of TNF. The results show that

the injectable in situ forming biodegradable PLGA system for the controlled delivery of the sp55-R, presented previously by in-vitro studies (22), was able to provide long-lasting protection against the harmful pathological effects of TNF in cachectic mice model. The effectiveness with which they block TNF action can perhaps be even further augmented using the injectable implants to specific targets those sites in the body where TNF is formed (e.g. the inflamed joints in rheumatoid arthritis, or intrathecally, for blocking the damaging effects of TNF in degenerative brain diseases, such as multiple sclerosis). Furthermore, this system is not limited only to the release of sp55-R, and may potentially be used to provide controlled release of other proteins to the site of need in-vivo.

REFERENCES

1. I. Olsson, M. Lantz, E. Nilsson, C. Peetre, H. Thysell, A. Grubb, and G. Adolf. Isolation and characterization of a tumor necrosis binding protein from urine. *Eur. J. Haematol.* **42**:270–275 (1989).
2. P. Seckinger, S. Isaaz, and J. M. Dayer. Purification and biologic characterization of a specific tumor necrosis factor α inhibitor. *J. Biol. Chem.* **264**:11966–11973 (1989).
3. H. Engelmann, D. Aderka, M. Rubinstein, D. Rotman, and D. Wallach. A tumor necrosis factor-binding protein purified to homogeneity from human urine protects cells from tumor necrosis factor toxicity. *J. Biol. Chem.* **264**:11974–11980 (1989).
4. H. Engelmann, D. Novick, and D. Wallach. Two tumor necrosis factor-binding proteins purified from human urine. *J. Biol. Chem.* **265**:1531–1536 (1990).
5. A. Kalinkovich, H. Engelmann, N. Harpaz, R. Burstein, V. Barak, I. Kalickman, D. Wallach, and Z. Bentwich. Elevated serum levels of soluble tumor necrosis factor receptors (sTNF-R) in patients with HIV infection. *Clin. Exp. Immunol.* **89**:351–355 (1992).
6. A. P. Cope, D. Aderka, M. Doherty, H. Engelmann, D. Gibbons, A. C. Jones, F. M. Brennan, R. N. Maini, D. Wallach, and M. Feldmann. Increased levels of soluble tumor necrosis factor receptors in the sera and synovial fluid of patients with rheumatic diseases. *Arthritis. Rheum.* **35**:1160–1169 (1992).
7. M. Lanz, S. Malik, M. L. Slevin, and I. Olsson. Infusion of tumor necrosis factor causes an increase in circulating TNF-binding proteins in humans. *Cytokine* **2**:402–406 (1992).
8. D. Aderka, A. J. Wysenbeek, A. Cope, H. Engelmann, Y. Molad, V. Hornik, Y. Levo, M. Feldmann, and D. Wallach. Correlation between serum levels of soluble tumor necrosis factor receptor and disease activity in systemic lups erythematosus. *Arthr. Rheum.* **38**:1111–1120 (1993).
9. I. Olsson, T. Gatanaga, U. Gullberg, M. Lantz, and G. A. Granger. Tumor necrosis factor (TNF) binding proteins (soluble TNF receptor forms) with possible roles in inflammation and malignancy. *Eur. Cytokine Netw.* **4**:169–180 (1993).
10. M. H. Bemelmans, D. J. Gouma, and W. A. Buurman. LPS-induced sTNF-receptor release in vivo in a murine model. Investigation of the role of tumor necrosis factor, IL-1, leukemia inhibiting factor, and IFN-gamma. *J. Immunol.* **151**:5554–5562 (1993).
11. P. Ettehadi, M. W. Greaves, D. Wallach, D. Aderka, and R. D. Camp. Elevated tumor necrosis-alpha (TNF-alpha) biological activity in psoriatic skin lesions. *Clin. Exp. Immunol.* **96**:146–151 (1994).
12. G. S. Vince, P. M. Starkey, R. Austgulen, D. Kwiatkowski, and C. W. Redman. Interleukin-6, tumor necrosis factor and soluble tumor necrosis factor receptors in women with pre-eclampsia. *Br. J. Obstet. Gynaecol.* **102**:20–25 (1995).
13. R. Eliaz, D. Wallach, and J. Kost. Long-term protection against the effects of tumor necrosis factor by controlled delivery of the soluble p55 TNF receptor. *Cytokine* **8**:482–487 (1996).
14. R. L. Dunn, G. L. Yewey, S. M. Fujita, K. R. Josephs., S. L. Whitman, G. L. Southard, W. S. Dernell, R. C. Straw, S. J. Withrow, and B. E. Powers. Sustained release of cisplatin in dogs from an injectable implant delivery system. *J. Bioactive and Compatible Polymers* **11**:286–300 (1996).
15. E. Tauboll, S. Lindstrom, W. Klem, and L. Gjerstad. A new injectable carbamazepine solution—antiepileptic effects and pharmaceutical properties. *Epilepsy Res.* **7**:59–64 (1990).
16. M. A., Crowther, A. Pilling, and K. Owen. The evaluation of glycofurol as a vehicle for use in toxicity studies. *Human & Experimental Toxicology* **16**:406 (1997).
17. E. Bechgaard, S. Gizurarson, and R. K. Hjortkjaer. Pharmacokinetic and pharmacodynamic response after intranasal administration of diazepam to rabbits. *J. Pharm. Pharmacol.* **49**:747–750 (1997).
18. W. Löscher and D. Hönack. Intravenous carbamazepine: comparison of different parenteral formulations in a mouse model of convulsive status epilepticus. *Epilepsia* **38**:106–113 (1997).
19. E. Bechgaard, S. Gizurarson, R. K. Hjortkjaer, and A. R. Sorensen. Intranasal administration of insulin to rabbits using glycofurol as an absorption promoter. *Int. J. Pharm.* **128**:287–289 (1996).
20. S. Gizurarson, G. Georgsson, H. Aggerbeck, H. Thorarinsdóttir, and I. Heron. Evaluation of local toxicity after repeated intranasal vaccination of guinea-pigs. Toxicology **107:**61–8 (1996).
21. *Handbook of Pharmaceutical Excipients,* Am. Phrmac. Assoc. and Pharm. Soc. of Gr. Br., 1994 p. 127.
22. R. E. Eliaz and J. Kost. Characterization of a polymeric PLGA injectable implant delivery system for the controlled release of proteins. *J. Biomed. Mater. Res.* **50**:388–396 (2000).
23. D. Aderka, H. Englemann, V. Hornik, Y. Skornick, Y. Levo, D. Wallach, and G. Kushtai. Increased serum levels of soluble receptors for tumor necrosis factor in cancer patients. *Cancer Res.* **51**:5602–5607 (1991).
24. A. Oliff, D. Defeo-Jones, M. Boyer, D. Martinez, D. Kiefer, G. Viocolo, A. Wolfe, and S. H. Socher. Tumors secreting human TNF/cachectin induce cachexia in mice. *Cell* **50**:555–563 (1987).
25. J. H. Korn, Y. Mory, A. Ziberstein, H. Holtmann, M. Revel, and D. Wallach. Cloning of genomic DNA for tumor necrosis factor and efficient expression in CHO cells. *Lymphokine Res.* **7**:349–358 (1988).
26. J. Murray, L. Brown, and R. Langer. Controlled release of microquantities of macromolecules. *Cancer Drug Delivery* **1**:119–23 (1984).