Protective Effect of 55- but not 75-kD Soluble Tumor Necrosis Factor Receptor-Immunoglobulin G Fusion Proteins in an Animal Model of Gram-negative Sepsis

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Summary

The aim of this study was to compare the ability of both a 55- and 75-kD soluble tumor necrosis factor receptor immunoglobulin G fusion protein (sTNFR-IgG) in protecting against death in a murine model of gram-negative sepsis. Pretreatment with 250 μg of the p75 construct delayed but did not avert death in this model, reducing peak bioactive TNF-α levels after infection from 76.4 ng ml⁻¹ in control mice to 4.7 ng ml⁻¹ in the treated group (p <0.05, two-sample t test). However, these low levels of bioactive TNF-α persisted in the p75 fusion protein–treated animals compared with the controls and were sufficient to mediate delayed death. In contrast, pretreatment with 200 μg of the p55 sTNFR-IgG gave excellent protection against death with complete neutralization of circulating TNF. Studies of the binding of TNF-α with the soluble TNFR fusion proteins showed that the p75 fusion construct exchanges bound TNF-α about 50-100-fold faster than the p55 fusion protein. Thus, although both fusion proteins in equilibrium bind TNF-α with high affinity, the TNF-α p55 fusion protein complex is kinetically more stable than the p75 fusion construct, which thus acts as a TNF carrier. The persistent release of TNF-α from the p75 fusion construct limits its therapeutic effect in this model of sepsis.

Despite significant advances in antibiotic treatment and intensive care management over the last 20 yr, the mortality from sepsis leading to multi-organ failure and septic shock has remained virtually unchanged (1, 2). Infection with a variety of different organisms can produce similar pathophysiologic changes within the host through the induction of a number of mediators. Principal among these is TNF-α, a cytokine produced mainly by activated macrophages, which is able in purified form to reproduce nearly all of the features of sepsis and septic shock (3, 4). Neutralization of TNF-α activity may thus be potentially beneficial in the treatment of this condition, and a number of different reagents designed to attenuate TNF-α action have been developed (5).

TNF-α exerts its effects through binding to high affinity cell surface receptors, of which there are two kinds, a 75-kD (p75) and a 55-kD (p55) form (6–8). These have significant sequence similarity in their extracellular domains, but differ completely in their intracellular portions (9). Many of the effects of TNF-α, including cytotoxicity, are produced by binding to the p55 receptor. This has been demonstrated by the use of agonist antireceptor antibodies (10, 11), and more recently by the use of mice with specific deletion of the p55 receptor gene (12, 13). The role of the p75 receptor is less well defined, but does include effects distinct from those of the p55 receptor, such as stimulating thymocyte proliferation (14). It has also been proposed that the p75 receptor may facilitate TNF-α binding to the p55 receptor, by initial rapid binding of TNF-α which is then subsequently passed to the p55 receptor (15).

TNF-α exists as a trimer in solution, and is potentially able to bind three receptor molecules (16–18). The mechanism by which receptor binding produces the cellular actions of TNF-α is not clear, but a number of experiments have shown that clustering of the p55 receptor is required for TNF-α effects mediated by this receptor (10). Both the p75 and p55 receptors also exist as soluble forms, produced by cleavage of the extracellular domains of the receptors (19, 20). These soluble receptors retain their high affinity binding for TNF-α but do not directly mediate any biological effects. Their production during sepsis may thus be a natural mechanism to attenuate TNF-α action (17, 21).

A number of reagents have been developed to neutralize TNF-α activity. mAbs to TNF-α have shown good activity in a number of animal models of experimental sepsis and are currently undergoing clinical trials (22, 23). Soluble TNF receptors (sTNFR) are an attractive means of attenuating
TNF action. They have high affinity binding not only for TNF-α, but also TNF-β, a form of TNF produced by activated T cells, which may be of importance in gram-positive infections with toxin-producing organisms (17, 21, 24). By the use of recombinant DNA technology, fusion proteins have been produced in which the soluble part of the TNFR is linked to a human IgG heavy chain constant region to form dimers through the intermolecular disulfide bridges joining normal IgG heavy chains. These dimeric fusion proteins are able to bind to the TNF trimer in two separate sites, thus binding with higher affinity than the natural soluble receptors (17, 21). In addition, the presence of the IgG heavy region confers a longer serum half-life for the fusion protein compared with the soluble receptor alone, with values in excess of 20 h (25, 26).

sTNFR or sfNFR-IgG fusion proteins have been tested in a number of different animal models of sepsis. In baboons challenged with live Escherichia coli, treatment with the p55 TNF was able to improve some of the hemodynamic abnormalities after bacterial challenge, with a suggestion of improved survival in treated animals, although the number of animals used was too small to demonstrate this conclusively (27). A p55 sfNFR-IgG fusion protein was able to protect against death in mice after challenge with LPS (26, 28). However, after intraperitoneal infection in mice, a p55 fusion protein was not able to improve survival, in common with other TNF neutralizing drugs in this particular model (29, 30). p75 fusion proteins have been tested in endotoxemia models of sepsis, where they have been shown to be protective against death in mice infected with LPS (21). However, in one case the fusion protein was shown to function as a carrier for TNF-α, although this did not seem to result in any deleterious effects (21). To better understand which TNF-IgG fusion protein might be more effective in the therapy of severe sepsis, we have tested the ability of both a p75 and a p55 fusion protein to protect against death in a model of sepsis in mice, using intravenous infection with live E. coli. We find that the p55, but not the p75, TNFR-IgG fusion protein was able to provide significant protection against death in this animal model of sepsis.

Materials and Methods

Animals. CD1 mice were used for all experiments and were obtained from Charles Rivers (Margate, UK). Animals weighing between 28 and 32 g were used for all experiments.

Materials. The sfNFR-IgG1 p75 fusion protein was kindly supplied by the Immunex Corporation (Seattle, WA). The sfNFR-IgG3 p55, sfNFR-IgG3 p75, and sfNFR-IgG1 p55 fusion proteins have been reported previously (17, 26, 28). The mAb to murine TNF-α (TN3) was kindly provided by Celltech (Slough, UK). Gentamicin was from Roussel Laboratories (Uxbridge, UK). All other materials were from Sigma (Poole, UK).

Bacteria. The bacterial strain used in all experiments was E. coli O111:B4 (kindly provided by Dr. Ben Appelmelk, Vrije Universiteit, Amsterdam, The Netherlands). For use in animal experiments, a single bacterial colony was inoculated into No. 2 broth (Oxoid, Basingstoke, UK) and grown for 5.25 h at 37°C. Bacteria were then harvested by centrifugation at 3,000 g for 15 min, washed once in sterile pyrogen-free saline, and resuspended in sterile pyrogen-free saline. Bacterial concentration was measured by absorbance at 325 nm and related to previous calibration curves for this organism.

Animal Model of Sepsis. This was performed as described (23). Briefly, animals were inoculated with an LD50 of E. coli by the tail vein; the inoculum was 3 × 106 CFU per animal. All animals received gentamicin injections intravenously at a dose of 1 mg kg⁻¹ at 2 and 5 h after infection, and on each subsequent day a further two i.v. doses at 1 mg kg⁻¹. Treatment with the p75 and p55 sfNFR-IgG fusion proteins was given 30 min before infection by i.v. injection; control animals were given either saline or an equivalent dose of human IgG. No difference in survival was seen in mice injected with either of these control treatments. Endotoxin levels of these protein solutions were <50 pg of endotoxin injected per animal. Mortality was recorded at regular intervals up to 72 h after infection; no mortality was observed after this time in the remaining animals. Blood for cytokine determinations was obtained from the cut tail tip at various times after infection. After clotting, serum was stored at −20°C until assayed.

Cytokine Determinations. TNF-α was measured by bioassay using actinomycin-sensitized L929 cells as described (31). Typically, the lower limit of sensitivity for this assay was ∼1 pg ml⁻¹. Serum samples were diluted 267-fold for assay, giving a detection limit in serum of ∼267 pg ml⁻¹.

LPS Assay. Endotoxin concentrations were obtained by Limulus amebocyte lysate assay using a kit according to the manufacturer’s instructions (Chromogenix, Möln达尔, Sweden).

Statistical Analysis. Survival curves were compared by the log rank test. Final survival percentages were compared by the two-sample t test. Differences in cytokine levels were compared at a given time point by the two-sample t test. A result was considered statistically significant if p < 0.05.

Dissociation of TNF-α from sfNFR-IgG Fusion Proteins. These experiments were performed with the p55 sfNFR-IgG3, p55 sfNFR-IgG1, and p75 sfNFR-IgG3 constructs reported previously (17, 26). 7.5 μg of the sfNFR-IgG fusion protein was incubated with 2.5 μg TNF-α containing 50 ng 125I-TNF-α (∼3 × 10⁶ cpm, iodinated according to [32]) in PBS for 20 min on ice. Unbound 125I-TNF-α was separated on a Superose 12 column (Pharmacia, Uppsala, Sweden) equilibrated in PBS. The 125I-TNF-α–sfNFR-IgG complexes were collected in a volume of 750 μl and treated with a 50-fold excess of unlabeled TNF-α at room temperature (23°C). 60-μl aliquots were withdrawn at different times and added to 10 μl packed protein G-Sepharose beads (Pharmacia) suspended in 40 μl PBS containing 2% FCS. After 4 min incubation with agitation, the beads were separated by filtering the sample through a 0.22-μm filter (MC filtration unit; Millipore, GYancourt, France). The radioactivity in the filtrate and on the filter was measured in a gamma-counter. The time required for quantitative precipitation of the fusion proteins with the protein G-Sepharose beads (4 min) was added to the overall incubation time.

Results

Effect of p75 sfNFR-IgG1 Fusion Protein on Survival. Groups of mice were pretreated either with 250 μg of the p75 sfNFR-IgG1 fusion protein or an equivalent volume of saline 30 min before an LD50 i.v. injection of E. coli. Survival in these two groups of animals is shown in Fig. 1. Control animals show a steady decrease in survival, starting at 5 h after infection, with a final survival percentage of 11%. The p75 sfNFR-IgG1–treated animals initially were protected against death,
with no deaths recorded until 20 h after infection. However, thereafter the mice began to die at the same rate as the control animals, with a final survival percentage of 30% in the p75 sTNFR-IgG1–treated group (Fig. 1). The overall difference in the survival curves between the two groups of mice was statistically significant (p < 0.05; log rank test), although the difference between the final survival percentages of 19% was not significant (95% confidence interval ± 22%). This delay in the death of infected mice produced by the p75 sTNFR-IgG1 protein, but with no final protective effect, was highly reproducible, with identical results obtained on two separate occasions. In addition, the same delayed death in p75 sTNFR-IgG1–treated mice persisted at this low level for the next 24 h, only becoming undetectable at 30 h after infection (Fig. 2).

**Figure 1.** Survival of mice following i.v. infection pretreated with 250 μg of the p75 sTNFR-IgG1 fusion protein (n = 20) 30 min before infection, or with saline (control, n = 18).

**Figure 2.** Bioactive serum TNF-α levels after i.v. infection in the animals from the experiment of Fig. 1. Each point is the mean value of sera from three mice. Error bars are ± 1 SEM. The dotted line indicates the lower limit of sensitivity of the cytotoxicity assay.

Role of TNF-α in the Delayed Death of the p75 sTNFR-IgG1 Animals. We wished to determine whether TNF-α was responsible for the delayed death of the p75 sTNFR-IgG1–treated mice after bacterial infection, as shown in Fig. 1. To answer this question, we set out to determine whether a neutralizing antibody to TNF-α with known effect in this model (23) could prevent the delayed death in the p75 sTNFR-IgG1–treated animals. Four groups of 10 mice were all simultaneously infected with an LD₀₀ of E. coli as before. Each group received a different treatment. Control–treated animals showed a progressive drop in survival, with a final survival percentage of 10% (Fig. 3, crosses). Mice receiving p75 sTNFR-IgG1 as before showed a delay in death, but with no final difference in outcome compared with the control group (Fig. 3, open circles). The remaining two groups of mice received a neutralizing antibody to TNF-α (TN3) at 4 h after infection (arrow in Fig. 3). When given on its own at this time after infection, this antibody is unable to protect mice from death (Fig. 3, filled squares), as we have previously shown (23). However, when TN3 was administered at this time in animals that had already received 250 μg of the p75 sTNFR-IgG1 fusion protein 30 min before infection, the anti-TNF-α antibody produced significant protection against death (Fig. 3, open squares; p < 0.05, log rank test). The low levels of bioactive TNF-α that persist in the circulation of the p75 sTNFR-
IgGl-treated animals are thus responsible for their delayed death.

The reduction in mortality produced by the administration of the TN3 in the p75 sTNFR-IgGl-treated mice is associated with a reduction in the bioactive TNF-α levels (Fig. 4). Animals receiving pretreatment with p75 sTNFR-IgGl still have measurable bioactive TNF-α levels of 2.2 ng ml⁻¹ (SEM 0.67) at 24 h after infection, compared with levels of 0.32 ng ml⁻¹ (SEM 0.32) in the mice receiving both the p75 and the TN3 (p < 0.05, two-sample t test).

**Effect of a Double Dose of the p75 sTNFR-IgGl Fusion Protein on Mortality.** One possible explanation for the lack of efficacy of the p75 sTNFR-IgG1 fusion protein in protecting against death is that an insufficient amount of the reagent was given. To address this question, we treated a group of mice with two doses of the p75 fusion protein: 250 μg was given 30 min before infection as before, and a further 250 μg dose was given at 4 h after bacterial inoculation. There was no difference in the survival of these mice receiving two doses of the p75 sTNFR-IgGl protein compared with control animals that were infected but that did not receive the fusion protein (data not shown). The animals receiving the double dose of the p75 sTNFR-IgGl protein still showed the low but persistent levels of bioactive TNF-α in the circulation, as seen with mice receiving a single dose (data not shown).

**Effect of p55 sTNFR-IgGl Fusion Protein on Survival.** The effects of the p55 sTNFR-IgGl fusion protein on survival following i.v. infection of mice with *E. coli* was investigated in exactly the same manner as with the p75 construct. Pretreatment of mice with 50 μg of the p55 sTNFR-IgGl protein 30 min before bacterial infection gave a significant protection from death compared with control untreated animals (Fig. 5; p < 0.05, log rank test). Animals treated with 200 μg of the p55 sTNFR-IgGl fusion protein produced an enhanced protective effect compared with the lower dose (Fig. 6). The difference in survival between p55 sTNFR-IgGl–treated and control mice was highly significant (p < 0.01, log rank test).

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**Figure 4.** Bioactive TNF-α serum levels in the animals from the experiment described in Fig. 3. Each point is the mean value of sera from three mice determined in cytotoxicity assays. Error bars are ± SEM.

**Figure 5.** Survival curves of mice after i.v. infection treated with either saline (Control) or with 50 μg of the p55 sTNFR-IgGl fusion protein at 30 min before infection. n = 20 for each group.

**TNF-α Levels after p55 sTNFR-IgGl Treatment.** The bioactive TNF-α levels in the serum of the mice in this experiment are shown in Fig. 7. At all time points after infection there was no detectable TNF-α in the serum of the p55 sTNFR-IgGl–treated mice, compared with the sharp peak of TNF-α seen at 90 min after infection in the control animals.

**Direct Comparison of the p75 and p55 sTNFR-IgGl Fusion Proteins.** To be certain that the observed differences between the p55 and p75 receptor constructs reflect a real difference in biological efficacy, we compared the ability of the two sTNFR-IgG reagents to protect against death directly within one experiment. A group of 30 mice was divided into three groups of 10 animals. One group received an i.v. injection of saline, another 250 μg of the p75 sTNFR-IgGl, and the remaining group 200 μg of the p55 sTNFR-IgGl. 30 min later, all animals were inoculated with an LD₉₀ of *E. coli*. 72-h survival in the three groups showed 2 out of 10 animals alive in the control group, 1 out of 10 alive in the p75 sTNFR-IgGl–treated group, and all 10 animals alive in the p55 sTNFR-IgGl–treated group.

**Kinetics of TNF-α Binding to p55 and p75 sTNFR-IgGl Fusion Proteins.** To investigate differences in the biochemical TNF-α binding properties of p55 and p75 TNF receptor–derived fusion proteins, the rate of exchange of TNF-α in
fusion protein–TNF-α complexes was determined (Fig. 8). Briefly, the various fusion proteins were complexed with 125I-TNF-α, placed in buffer containing excess unlabeled TNF-α, and the time-dependent release of 125I-TNF-α was measured. These studies revealed that TNF-α complexed with the p75 sTNFR-IgG exchanged at a significantly higher rate than when complexed with the p55 sTNFR-IgG, as shown by the half-lives of about 7 min for the p75 sTNFR-IgG–TNF-α complex and about 8 h for the p55 sTNFR-IgG–TNF-α complex (Fig. 8).

Discussion

We have shown in the experiments described in this paper that there is a significant difference in the behavior of the p75 and p55 sTNFR-IgG1 fusion proteins in their ability to neutralize TNF-α and protect against death in a murine model of gram-negative sepsis. The p75 construct is able to attenuate the high peak levels of bioactive TNF-α produced after inoculation of mice with E. coli, but thereafter these low levels of TNF-α persist in the circulation for many hours and mediate the late death of the mice. The p55 construct, on the other hand, produces complete neutralization of serum TNF-α at all time points after infection, and provides good protection against death in this model of sepsis. The beneficial effect of the p55 sTNFR-IgG construct on survival compared with the p75 construct was highly reproducible between experiments and, importantly, could be demonstrated when the two reagents were compared directly within one experiment.

The delay in death produced by the p75 sTNFR-IgG1 fusion protein in the model used in the experiments described here was sufficient to produce a statistically significant difference between the survival curves for treated and control groups of mice, although the final outcome was similar between the two groups and not significantly different (Fig. 1). The bioactive TNF-α levels in these animals provide an explanation for this result. The p75 sTNFR-IgG1 protein is able to attenuate the peak TNF-α levels after bacterial inoculation, but thereafter acts as a carrier for TNF-α, which persists in the circulation at low levels until 30 h after infection, in contrast to the very rapid disappearance of TNF-α in the control animals (Fig. 2). This ability of the p75 sTNFR-IgG1 protein to act as a carrier for TNF-α has been observed in mice after experimental endotoxemia (21).

The release of bioactive TNF-α from the p75 sTNFR-IgG1 protein to produce low but persistent levels of this cytokine up to 30 h after infection is sufficient to produce the delayed death in these animals. This is demonstrated by the ability of a neutralizing mAb to TNF-α (TN3) administered 4 h after infection to protect the p75 sTNFR-IgG1–treated animals (Fig. 3). When administered at this time point, the TN3 is ineffective at protecting against death on its own, presumably because the peak of TNF-α has already passed (Fig. 4, control animals). The TN3 antibody also reduces the circulating bioactive TNF-α levels compared with the mice receiving the p75 sTNFR-IgG1 fusion protein alone (Fig. 4).

Why does the TNF-α carried by the p75 sTNFR-IgG fusion protein produce a deleterious effect in the model of gram-negative sepsis described here, but is not harmful after endotoxin challenge in mice (21)? There are several possible explanations. The TNF-α levels produced after bacterial challenge in the model used in our experiments are much higher. Mice typically have peak levels of ∼80 ng ml⁻¹ after bacterial infection (Fig. 2) compared with the levels of ∼0.5 ng ml⁻¹ reported after endotoxin challenge in mice (21). In addition, i.v. challenge with live bacteria is a considerably more complex stimulus than LPS challenge alone. For example, the LPS-resistant mouse strain C3H/HeJ is as susceptible to i.v. challenge with live E. coli as its parent strain, C3H/HeN, despite an enormous difference in susceptibility after LPS challenge (33). Finally, in producing a lethal effect in animals, TNF-α synergizes strongly with other cytokines such as IFN-γ which are produced at high levels in the model of sepsis used in the experiments reported here (34, and data not shown).

An important consideration in the experiments with the p75 sTNFR-IgG protein is that the results obtained might be dependent on the exact stoichiometry of binding of the p75 fusion protein with TNF-α. In vitro, the carrier functions of soluble TNFR have been demonstrated at low ratios of receptor to TNF; at higher soluble receptor concentrations, the neutralizing properties predominate (35). However, the administration of a second identical dose of the p75
fusion protein at 4 h after infection in animals that had already received 250 µg 30 min before bacterial inoculation did not improve survival. In addition, this double dose of p75 TNFR-IgG1 fusion protein did not alter substantially the prolonged presence of bioactive TNF-α in the serum of the infected animals. This is in marked contrast to the effect of TN3 described above and shows that prolonged presence of bioactive TNF-α in the serum of the p75 TNFR-IgG1-treated mice is not due to inadequate dosing of the fusion protein.

In contrast to the p75 TNFR-IgG1 fusion protein, the p55 TNFR-IgG1 protein provides good protection against death in this model of sepsis (Fig. 6). The protective effect is dependent on the dose of the administered p55 material. At 50 µg per mouse, the protective effect was much more modest than that seen with a 200 µg per animal dose (Figs. 5 and 6). With a dose of 200 µg of p55 TNFR-IgG1 protein given 30 min before infection, complete neutralization of circulating bioactive TNF-α was produced (Fig. 7). The specificity of the beneficial effect on survival of the p55 reagent is shown by the lack of benefit seen in animals injected with either saline, human IgG, or the p75 TNFR-IgG1 construct.

Why should there be this difference between the p55 and p75 reagents? In many respects the p55 and p75 TNFR-IgG fusion proteins have similar properties. They both bind TNF-α in solution with similar high equilibrium binding constants (17, 21). The elimination half-life of both reagents is very similar, in the order of 20 h (25, 26). However, one possible explanation for their different effects in this model of sepsis is their different kinetics of TNF-α binding and release. The p75 TNFR-IgG fusion protein binds and releases TNF-α ~100-fold faster than the p55 fusion protein (Fig. 8). The different binding kinetics of p55 and p75 TNFR-IgG reflect inherent properties of the p55 and p75 TNFR molecules that carry over into the fusion protein constructs (36, and Loetscher H., D. Belluccio, and W. Lesslauer, unpublished data). Thus, although the p75 fusion protein under equilibrium conditions has the same affinity as the p55 construct, it is less kinetically stable. This has a profound influence on the partitioning of TNF-α between fusion protein, natural soluble and membrane bound TNFR in blood, as reflected by the different TNF-α concentrations revealed in the cytotoxicity assays. The difference in outcome of the p55 and p75 TNFR-IgG1 treatments thus may be understood from the different binding kinetics of the two constructs.

What are the therapeutic implications of these results? Results from animal models must be interpreted cautiously before extrapolation to human disease. However, the experiments described here do demonstrate an important difference in the biological properties of the two TNFR-IgG fusion proteins. The protective effects of the p55 construct compared with the p75 protein in the model of sepsis used in our experiments suggest that the p55 TNFR-IgG will also be more likely to be effective in human disease.

This work was supported by the Medical Research Council, UK through the award of a Medical Research Council Clinician/Scientist Research fellowship to T. J. Evans.

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Received for publication 22 June 1994 and in revised form 15 August 1994.

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