Development of a novel, nonimmunogenic, soluble human TNF receptor type I (sTNFR-I) construct in the baboon

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As the potential application for anti-TNF-α therapies has shifted from acute to chronic inflammatory diseases, however, concerns have arisen regarding the antigenicity, safety, and altered pharmacokinetics of these constructs when administered repeatedly over extended periods of time. A common approach for inhibiting the actions of TNF-α has been to employ protein constructs composed of the extracellular domains of either the p55 type I (sTNFR-I) or p75 type II sTNFR (sTNFR-II). Several years ago, our laboratory reported that infusion of the human sTNFR-I could bind TNF-α in vivo and attenuate the inflammatory response to a lethal bacterial challenge (24). However, use of native sTNFR-I was limited by the short half-life of the monomeric form and the relative instability of the sTNFR-I/TNF-α plasma complexes that were formed.

Two different approaches have been employed to increase the biological half-life of sTNFR-I and its capacity to neutralize homotrimetric TNF-α. By fusing the extracellular domains of either sTNFR-I or sTNFR-II to the hinge and Fc region of a human immunoglobulin, novel immunoadhesins have been constructed with specificity for TNF-α and the pharmacokinetics of an immunoglobulin (2). An alternative approach has been to modify the amino acid sequence of the sTNFR-I through site-directed mutagenesis and to covalently bind the soluble receptor to polyethylene glycol (PEG). Our laboratory has recently demonstrated that constructs composed of two sTNFR-I co-

immunoadhesin; sepsis; pharmacokinetics; inflammation

PROTEIN-BASED THERAPIES AIMED at inhibiting the actions of tumor necrosis factor (TNF)-α currently include monoclonal antibodies and soluble TNF receptor (sTNFR) constructs. These inhibitors have been or are presently undergoing clinical evaluation in patients with a variety of acute and chronic inflammatory diseases, such as sepsis syndromes, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and congestive heart failure. Although anti-TNF-α therapies have not proven successful in acute systemic inflammatory syndromes (1, 11), these same therapeutic approaches have shown efficacy in patients with rheumatoid arthritis, inflammatory bowel disease, and congestive heart failure (7, 17, 23) and are approved for clinical use in rheumatoid arthritis and inflammatory bowel disease.
valently linked to PEG [dimeric sTNFR-I or TNF binding protein (TNF-bp)] have extended plasma half-lives (20–30 h vs. 20 min) and increased TNF-α neutralization activity compared with native, unPEGylated sTNFR-I (9, 20).

Although these initial efforts have resulted in constructs with much longer plasma half-lives and increased biological activity, immunogenicity has remained a problem. In clinical trials with the administration of an humanized monoclonal antibody against TNF-α, patients frequently developed antibodies; the appearance of antibodies was associated with increased plasma clearance on repeated administration (10). Similarly, an sTNFR-I immunoadhesin was withdrawn from clinical trials in rheumatoid arthritis in part because of concerns regarding its immunogenicity (5, 15), although a similar sTNFR-II immunoadhesin has been shown to be only modestly immunogenic (17). A dimeric, PEGylated sTNFR-I has also been evaluated in patients with rheumatoid arthritis, and, although the construct has shown efficacy, immunogenicity has remained a significant problem (18).

In a previous report, our laboratory examined the plasma half-life and immunogenicity of three different PEGylated dimeric sTNFR-I constructs that differed only in the number of functional domains of the sTNFR-I present (20). Native sTNFR-I contains four functional domains, and recombinant proteins were generated that contained 4, 3, or 2.6 functional domains (20). All three constructs were equally effective in neutralizing TNF-α and protecting the juvenile baboon from lethality in bacteremic shock. Although all of the dimeric constructs were immunogenic, the degree of immunogenicity increased with the number of sTNFR-I functional domains. Furthermore, plasma clearance of the constructs was most rapid in those animals that developed antibodies from the previous injection (20).

The present study was a continuation of this investigational approach. In the present report, we have evaluated the pharmacokinetics and immunogenicity of six third-generation sTNFR-I constructs in the healthy baboon at clinically relevant doses, in an effort to minimize immunogenicity and maintain their in vivo capacity to neutralize TNF-α. Five of the constructs were PEGylated monomeric or dimeric sTNFR-I, which varied in their number of functional domains (2.6 or 4.0). These PEGylated sTNFR-I were compared with a 4.0-domain sTNFR-I immunoadhesin, similar to one that our laboratory previously reported (25). In the second phase of the study, a PEGylated monomeric sTNFR-I construct shown to be nonimmunogenic in the first phase was compared with a dimeric sTNFR-I construct for its ability to neutralize TNF-α at doses previously shown to protect against lethal bacteremic shock in the naive baboon. Finally, the baboon sTNFR-I was cloned, and its sequence was compared with human sTNFR-I in an effort to extrapolate these results from the nonhuman primate to the clinical setting.

Table 1. Treatment groups and the sTNFR-I constructs employed

<table>
<thead>
<tr>
<th>Group</th>
<th>TNFR Domains</th>
<th>Monomeric/Dimeric Construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4.0</td>
<td>Dimeric Immunoadhesin</td>
</tr>
<tr>
<td>II</td>
<td>4.0</td>
<td>Monomeric 30-kDa PEG (N105)</td>
</tr>
<tr>
<td>III</td>
<td>4.0</td>
<td>Monomeric 30-kDa PEG (C105)</td>
</tr>
<tr>
<td>IV</td>
<td>2.6</td>
<td>Monomeric 30-kDa PEG (N105)</td>
</tr>
<tr>
<td>V</td>
<td>4.0</td>
<td>Dimeric 20-kDa PEG (C105)</td>
</tr>
<tr>
<td>VI</td>
<td>2.6</td>
<td>Dimeric 20-kDa PEG (C106)</td>
</tr>
</tbody>
</table>

Soluble tumor necrosis factor receptor p55 type I (sTNFR-I) constructs were evaluated in experimental study 1. Construct I, a 4.0-domain immunoadhesin, served as a control. Groups II–VI represent PEGylated monomeric or dimeric sTNFR-I, which vary in the number of functional domains (2.6 or 4.0). N105 refers to attachment of polyethylene glycol (PEG) at the amino (N) group of AA105. The C105 construct is attached at the cysteinyl (C) group of AA105. Constructs IV–VI are attached at the C group of AA105 or AA106.
containing 10 mM Tris-HCl, pH 8.8, 10 mM KCl, 0.002% (vol/vol) Tween 20, and 6 units of UTma DNA polymerase. The reaction was cycled 25 times using a Perkin Elmer 9600 thermal cycler (95°C for 45 s, 63.1°C for 1 min, and 72°C for 2 min). The products were gel purified on a 3% NuSieve gel (FMC). Bands of the correct size were excised and purified using a Promega Wizard PCR prep purification column. The fusion PCR was then accomplished using the same reaction conditions except that a 2-µl aliquot of each gel-purified product from the above-described reactions was used, and no primers were added to the reaction. This reaction was cycled 10 times using the same cycling protocol. Finally, amplification of the desired immunoadhesin gene product was achieved by PCR in which 1 µl of the fusion reaction and 200 pmol of each P1 and P4 were cycled 25 times under identical conditions. The product was blunt-end cloned into PCRScript (Promega). Positive clones were screened by restriction digest and sequenced to confirm the desired sequence. The resultant immunoadhesin gene was restriction digested with Hind III and Sal I and cloned into a mammalian expression vector. It was then overproduced in Chinese hamster ovary cells and purified by ion exchange chromatography and affinity chromatography.

 Constructs II [4.0 domain, 30-kDa PEG sTNFR-I (N105) monobell], III [4.0 domain, 30-kDa PEG sTNFR-I (C105) monobell], and IV [2.6 domain, 30-kDa PEG sTNFR-I (N105) monobell] were analogs that were composed of either the 4.0 or 2.6 domains of the sTNFR-I, with a single amino acid substitution required for PEGylation. The 4.0-domain constructs (constructs II and III) contained the sequence from MetO to Asn143, and 2.6-domain construct (construct IV) contained the amino acid sequence from MetO to Leu108 (16). The PEG monomers of 4.0-domain sTNFR-I were modified either with 30-kDa PEG aldehyde through the alpha-amino group of AA108 (N105; construct II), or through the free cysteinyl residue (C105; construct III). The PEG monomer of 2.6-domain sTNFR-I was modified at the alpha-amino group (N105; construct IV) with the 30-kDa PEG aldehyde.

 Constructs V and VI, which are dimeric forms of the sTNFR-I containing either the 2.6 domain or 4.0 domain (e.g., TNF-bp), have been described elsewhere (9, 13, 14, 19, 21). The cloning and expression of constructs II–VI were performed essentially as described previously (20). The dimer constructs were covalently linked with 20-kDa PEG bis-vinyl sulfone, which links the proteins via free sulfhydryl groups at positions C105 and C106 for the 4.0-domain and 2.6-domain forms, respectively. Very briefly, constructs II–VI were produced in inclusion bodies in Escherichia coli. The proteins were solubilized with 6 M urea and refolded out of a dilute urea solution. The refolded protein was purified using ion exchange and hydrophobic interaction chromatography. After the ion-exchange purification step, the protein solutions were concentrated and diafiltered into an appropriate buffer and sterile filtered with a 0.2-µm Durapore membrane. Constructs II and IV were modified with PEG 30-kDa aldehyde at the alpha-amino group via reductive alkylation. Constructs V and VI were PEGylated with PEG 20-kDa bis-vinyl sulfone to make sTNFR-I dimers. Constructs III, V, and VI were reduced before attachment of the PEG with 4 mol dithiotreitol per 1 mol of protein at 5–6°C. All reactions were performed in the presence of 30% glycerol. All six constructs were obtained from Amgen. Endotoxin concentrations of all preparations were found to be <0.2 endotoxin U/mg protein.

 Molecular cloning of primate TNFR-I soluble domain. Peripheral blood from baboons (Biomedical Resources Foundation, Houston, TX) was collected into heparinized tubes and stored at 4°C. The lymphocyte fraction of whole blood was isolated by centrifugation over density gradients (Ficoll-Paque Plus, Sigma Chemical, St. Louis, MO), following the manufacturer’s recommended procedures. The resulting nucleated cells were washed in sterile phosphate-buffered saline and then lysed in guanidine thiocyanate (Pharmacia, Piscataway, NJ). Total cellular RNA was isolated by layering the cell lysates onto cesium trifluoroacetate density gradients (Pharmacia) and then centrifuging them at 30,000 rpm for 24 h at 15°C. The RNA pellets were resuspended in RNAse-free distilled water and then analyzed by gel electrophoresis to check integrity.

 The protein coding region spanning the extracellular domain of baboon TNFR-I was amplified from peripheral blood total RNA by RT-PCR using the following primer pairs derived from the human TNFR-I sequence.

 Exons 1–4 5′-GCC GCT GGT GCT CCT G
 Exons 3–6 5′-CCG GCC ACC ATA CGG ACT G
 3′-GGGGCCA ACA GCA CTG TGD TTG TG

Primers (0.4 µM final) were added to ~500 ng of RNA in EZ RTth7 RNA Core reaction buffer (Perkin-Elmer, Foster City, CA) and annealed at 60°C for 30 min. Primer extension proceeded for 1 min at 94°C, followed by 40 amplification cycles at 60°C for 30 s and 94°C for 15 s, and one cycle at 60°C for 7 min. The PCR products obtained were then isolated by gel electrophoresis and subcloned into the plasmid vector pCDNA (Invitrogen, La Jolla, CA). Isolated recombinant clones and the purified RT-PCR amplification products were sequenced as previously described (19). The protein coding frame of the contiguous cDNA sequence was deduced, and the 193-amino acid residues of the baboon sTNFR-I proteins were aligned with the corresponding human sequence using the Pretty Plot application (GCG Program, University of Wisconsin) as previously described (19).

 In vitro TNF-α neutralization activity of the individual construct. The ability of each of the six different sTNFR-I constructs to neutralize TNF-α bioactivity was initially determined in a cell-based culture system. Very briefly, WEHI 164 clone 13 cells were incubated in 96-well microtiter plates with RPMI-1640 medium with 10% heat-inactivated fetal calf serum and 1 µg/ml streptomycin at 37°C with 5% CO2. Added to each well were increasing quantities of one of the six sTNFR-I constructs, ranging in concentration from 10 pg/ml to 100 µg/ml. Ten minutes later, 600 pg/ml of recombinant human TNF-α were added to each well, and cells were incubated an additional 18 h. Over the last 6 h, cell viability was determined by the addition of 6 mg/ml of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. After removal of the medium, cells were lysed with isopropanol and diluted with distilled water. Absorbance at 570 and 690 nm was used to estimate the number of viable cells remaining.

 Experimental study 1. The initial baboon study was aimed at determining the immunogenicity and pharmacokinetics of the six different sTNFR-I constructs when repeatedly administered to healthy, juvenile baboons at doses that approximated their potential use in patients. Eighteen juvenile female baboons (Papio anubis; 2.0–2.9 kg) were purchased from Biomedical Research Foundation (Houston, TX). All animals were quarantined for a period of at least 4 wk at the Animal Resource Center of the University of Florida College of Medicine to confirm their good health and lack of transmissible disease. The studies were approved by the Institu-
tional Animal Care and Use Committee of the University of Florida.

On day 0, after an overnight fast, all of the animals were anesthetized and instrumented, as previously described (20). During a 1-h equilibration period, hemodynamic parameters and blood samples were obtained. The animals were randomly assigned to one of six treatment groups, wherein each baboon received a bolus (30 s) intravenous infusion of 0.2 mg/kg of one of the six sTNFR-I constructs. The dose of the sTNFR-I constructs was chosen to approximate the quantities administered to patients [0.03, 0.10, and 0.30 mg/kg body wt (BW)] as a therapy for rheumatoid arthritis (18). In this clinical study, construct V had been administered to patients with refractory rheumatoid arthritis every 3 wk, and, although some efficacy was seen, significant immunogenicity was also observed. The present study was designed to mimic the clinical study and to determine whether any of the constructs had altered pharmacokinetics and immunogenicity.

After dosing on day 0, the animals were observed for 8 h, during which time they received 0.9% sodium chloride (3 ml·kg⁻¹·h⁻¹) as maintenance fluid. At the end of the 8-h period, all catheters were removed, and the animals were returned to their cages after receiving analgesia.

On days 21 and 42, the baboons were reanesthetized and, after collection of a baseline venous blood sample, each animal was administered the same dose (0.2 mg/kg) of the same protein as on day 0.

Blood sampling for pharmacokinetics. Venous blood samples were obtained predose, at 15 min, and on days 1, 2, 3, 5, 8, 11, 16, and 21 after each of the three intravenous injections (given on days 0, 21, and 42). All samples were anticoagulated with EDTA or heparin and cooled on ice immediately after drawing. The plasma fraction was separated by centrifugation and stored at −80°C until assay.

Plasma sTNFR-I dimer ELISA analysis. Determination of plasma levels of each construct was performed by using an antigen capture ELISA developed using affinity-purified polyclonal antibodies to the 4.0-domain construct. The antibody was raised against recombinant human sTNFR-I in the goat and was affinity purified using dimeric 4.0-domain sTNFR-I. For each construct, plasma samples were run against a standard curve of the same test article. Ninety-six-well plates were coated with 4.0 mg/ml of goat anti-4.0-domain sTNFR-I in the goat and was affinity purified using dimeric 4.0-domain sTNFR-I. After washing, the samples and the respective sTNFR-I construct standards (diluted in 20% goat serum in Dulbecco’s phosphate-buffered saline, containing 0.05% Tween 20) were placed on the plate. After washing, the biotinylated primary antibody (goat antihuman dimeric sTNFR-I) was added to the plate. After washing again, streptavidin-horseradish peroxidase was added. The substrate solution contained 2.5 mg/ml 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and 0.05% H₂O₂. The plates were read at 405–490 nm on a Molecular Devices Vmax ELISA plate reader and fit to a four-parameter equation by Softmax software. The parameters from the equation were used to calculate the respective construct sTNFR-I concentrations in plasma samples. The lowest limit of detection for all sTNFR-I constructs in the assay, when corrected for dilution, was 0.490 ng/ml. All samples were baseline corrected using the prestudy value for each individual to account for endogenous levels of sTNFR-I. To ensure accuracy in the reported plasma levels of each construct in antibody-positive animals, plasma samples were analyzed at a variety of dilutions to ensure linearity.

Anti-sTNFR-I IgG antibody analysis. The plasma samples were evaluated for the presence of anti-sTNFR-I antibodies using an antibody-capture enzyme-linked immunosassay technique. Briefly, 96-well plates were coated with capture antigen (1 µg/ml) by incubating overnight at 4°C. The capture antigen for each construct is summarized in Table 2.

Nonspecific protein binding sites were blocked with 2% BSA. Duplicate test plasma and positive control (goat anti-serum to sTNFR-I dimer) were diluted 1:50 in 20% normal goat serum and added to the plate. After an incubation of 1.5 h at 37°C, an alkaline phosphatase-secondary antibody (alkaline phosphatase anti-baboon IgG) was added to wells containing test plasma to detect captured antibodies. Color development was with the p-nitrophenylphosphosphate substrate system. All wells were washed between steps with PBS-0.05% Tween 20. A plate reader was used to monitor optical density (OD) between 405 and 490 nm.

In order for a sample to be considered reactive, the mean OD of the postdose sample must have been at least twice the mean OD of that animal’s predose sample (i.e., postdose-to-predose ratio > 2). Any reactive sample, along with its predose sample, was titered (serially diluted) at a starting dilution of 1:50. The dilution that was twice the background (blank wells) was considered the titer of the reactive postdose sample.

Noncompartmental analysis. The parameters defining the plasma concentration time profile characteristics were determined by noncompartmental analysis using WinNonlin (version 1.1, Scientific Consulting, Lexington, KY).

Experimental study 2. The second study was aimed at determining the relative efficacy of one of the third-generation sTNFR-I constructs to neutralize TNF-α in vivo and prevent lethality against E. coli-induced bacteremic shock, compared with a control dimeric construct (construct V) that had previously been shown to be effective at this dose (9, 20). Because of the high levels of TNF-α produced in response to the intravenously administered E. coli, the quantities of sTNFR-I required to protect the animals are much greater than are those required to treat disease progression in rheumatoid arthritis. Fifteen juvenile baboons were studied. Animals were quarantined and instrumented as described in Experimental study 1. After the animals were equilibrated for 1 h, they were randomized to one of the five treatment groups to receive either placebo (n = 3); 1 mg/kg BW of a dimeric 4.0-domain sTNFR-I (construct V) (n = 3); or 1, 5, or 10 mg/kg BW of a monomeric 2.6-domain sTNFR-I (construct IV) (n = 3 each).

Table 2. Constructs and capture antigens used to detect plasma concentrations

<table>
<thead>
<tr>
<th>Group</th>
<th>Construct</th>
<th>Capture Antigen</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>4.0-domain sTNFR-I immunoadhesin</td>
<td>4.0-domain monomeric sTNFR-I (N105)</td>
</tr>
<tr>
<td>II</td>
<td>4.0-domain monomeric sTNFR-I (N105)</td>
<td>4.0-domain monomeric sTNFR-I (N105)</td>
</tr>
<tr>
<td>III</td>
<td>4.0-domain monomeric sTNFR-I (C105)</td>
<td>4.0-domain monomeric sTNFR-I (C105)</td>
</tr>
<tr>
<td>IV</td>
<td>2.6-domain monomeric sTNFR-I (N105)</td>
<td>2.6-domain monomeric sTNFR-I (C105)</td>
</tr>
<tr>
<td>V</td>
<td>4.0-domain dimeric sTNFR-I (C105)</td>
<td>4.0-domain dimeric sTNFR-I (C105)</td>
</tr>
<tr>
<td>VI</td>
<td>2.6-domain dimeric sTNFR-I (C106)</td>
<td>2.6-domain dimeric sTNFR-I (C106)</td>
</tr>
</tbody>
</table>
The proteins were administered intravenously into the cephalic vein. Subsequently, lethal *E. coli* bacteremia was induced in the experimental animals by administration of \(10^{10–11}\) colony-forming units/kg of live *E. coli*. The live *E. coli* bacteria were infused over 30 s into the femoral vein. This quantity of bacteria represents an approximate 100% lethal dose of live *E. coli*. All investigators performing the study were blinded as to the experimental treatment regimen. Continuous infusions of physiological saline (0.9% sodium chloride; 3 ml·kg\(^{-1}\)·h\(^{-1}\)) were administered via the antecubital fossa to all animals for 8 h. In addition, *E. coli* and 48 h, and on received appropriate analgesics (buprenorphine, 0.02 mg/kg BW of cephtriaxon. The animals were returned to their cages, this time and at 24 h, the intramuscular injection of 30 mg/kg BW of saline was administered. The animals were allowed to emerge from anesthesia.

Blood samples were obtained from the baboons at specified times (0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, and 8 h for cytokines, pharmacokinetics, and total and differential white blood cell counts).

Before the animals were returned to their cages, the baboons received intramuscular 0.01 mg/kg BW of buprenorphine as an analgesic. In addition, the animals received at this time and at 24 h, the intramuscular injection of 30 mg/kg BW of cephtriaxon. The animals were returned to their cages, and subsequent survival to the lethal bacterial challenge was evaluated for 14 days. During this period, the animals received appropriate analgesics (buprenorphine, 0.02 mg/kg BW im every 24 h) to eliminate or minimize discomfort. At 24 and 48 h, and on days 3, 5, 8, 11, and 14, the animals were briefly anesthetized with ketamine HCl (10 mg/kg BW im), and venous blood samples were obtained.

Multisystem organ failure developed in some of these animals, necessitating a premature euthanasia, as determined by the Institutional Animal Care and Use Committee, University of Florida. Euthanasia was performed in any animal that was deemed by the clinical veterinarians (blinded to the treatment groups) to be suffering excessive discomfort and unlikely to recover. Excessive discomfort that required the termination of the study included any of the following: 1) failure to maintain a sitting or upright position over the previous 12 h; 2) failure to take food or water within the previous 12 h; 3) uncontrollable bleeding from catheter sites; and 4) unresponsiveness to external stimuli. Animals that met any of these criteria were promptly killed by a pentobarbital sodium overdose (150 mg/kg BW iv). Animals euthanized for animal welfare reasons were deemed to be nonsurvivors.

Animals surviving 14 days were anesthetized with 10 mg/kg ketamine HCl, a venous blood sample was obtained, and baboons were euthanized as described above.

Analytic methods. Plasma TNF-α activity was determined by both ELISA and plasma-based bioassay. The TNF-α sandwich ELISA employs a monoclonal antibody as the capture and a polyclonal rabbit anti-TNF antiserum as the secondary antibody. The ELISA can recognize both free TNF-α and TNF-α bound to either of its sTNFRs (24), although the affinity for TNF-α bound to its shed receptor is reduced. The decreased affinity was estimated by analyzing known quantities of TNF-α in the ELISA with concentrations of the different sTNFR-I construct at levels found in the baboon plasma.

TNF-α bioactivity was assessed by using the WEHI 164 clone 13 cytotoxicity assay (21), which detects only bioactive protein. Interleukin (IL)-1β and IL-6 concentrations were measured by ELISA as previously described (25).

Hematology and biochemical measurements. Complete blood counts were performed at defined intervals, and the number of circulating leukocytes per cubic millimeter was determined by Coulter counter (Coulter Electronics, Hialeah, FL). Platelet counts were determined in the same manner. Differential counts were obtained by examining at least 100 cells on a Wright stained peripheral blood smear.

Statistical analyses. Data are presented as means ± SD or SE. Differences in pharmacokinetics among the treatment groups at the same time points were determined by one-way analysis of variance. Changes in the pharmacokinetics with repeated injections of the constructs were analyzed by paired t-test. Differences in hemodynamics in response to *E. coli* and the different constructs were determined by two-way analysis of variance (time vs. treatment). Post hoc comparisons were performed by using the Student-Newman-Keuls multiple-range test. Significance was determined at the 0.05 level of confidence.

RESULTS

In vitro neutralization studies. All of the constructs effectively neutralized TNF-α bioactivity as determined in a cell-based bioassay (Fig. 1). The dimeric constructs (constructs I, V, and VI), including the dimeric immunoadhesin, were most effective at neutralizing TNF-α. Approximately 50% neutralization of TNF-α bioactivity (to 600 pg/ml of TNF-α) was achieved with 5–10 ng/ml of each of the constructs. In contrast, the monomeric constructs (constructs II, III, and IV) were ~20-fold less effective at neutralizing TNF-α bioactivity on a weight basis, with concentrations on the order of 100–200 ng/ml required to produce 50% neutralization of TNF-α.

Study 1: Physiological analyses. Administration of all six sTNFR-I constructs to the healthy naive pri-

![Fig. 1. In vitro inhibition of tumor necrosis factor (TNF-α) bioactivity by TNF receptor constructs. WEHI 164 clone 13 cells (5 x 10^4/ml) were plated into 96-well microtiter plates with increasing quantities (10 pg/ml through 100 μg/ml) of one of the TNF receptor constructs. Ten minutes later, 600 pg/ml of recombinant human TNF-α were added to each well, and cell survival was evaluated 18 h later. Over the last 4 h, 6 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added to each well, and cell no. was determined by absorbance at 570 and 690 nm. • Group I (4.0-domain immunoadhesin); ▲ group II (4.0 domain, monomeric 30-kDa N105); ▲ group III (4.0-domain monomeric C105); × group IV (2.6 domain, monomeric N105); ● group V (4.0 domain, dimeric C105); ● group VI (2.6 domain, dimeric C106), sTNFR, soluble TNF receptor.

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mutes at a dose of 0.2 mg/kg BW was without any acute hemodynamic or hematological effect, as evaluated over the initial 8 h of constant monitoring. Mean arterial blood pressure, heart rate, core temperature, and urine output were unaffected by treatment with any of the constructs (data not shown). Furthermore, on days 21 and 42, when the animals were re-injected with additional quantities of the same constructs as administered on day 0, no adverse physiological responses were noted. Thus the constructs appeared to be safe in the otherwise healthy, anesthetized baboon.

Study 1: Pharmacokinetic analyses. The parameters describing the plasma half-life of the six groups are shown in Table 3. The elimination half-life did not differ significantly among any of the PEGylated sTNFR-I groups (constructs II–VI) after the first injection, although it did tend to be longer for the monomeric constructs. The half-life of the sTNFR-I immunoadhesin was significantly longer than any of the PEGylated constructs (P < 0.05).

The major change in the pharmacokinetics after the second and third administration occurred in baboons receiving either the 4.0-domain dimeric construct (construct V) or the sTNFR-I immunoadhesin (construct I). In these two groups, the terminal half-life significantly decreased with repeated injections. The decrease in terminal half-life in baboons receiving the 4.0-domain dimeric sTNFR-I was greater than 50 and 80% (P < 0.01) after the second or third injections, respectively, whereas the decrease in the baboons receiving the immunoadhesin was >90% (P < 0.01). In contrast, the terminal half-life of the other PEGylated sTNFR-I did not statistically change with repeated injections.

Study 1: Antibody responses. All of the constructs were immunogenic in the baboon with the exception of the 4.0-domain dimeric sTNFR-I immunoadhesin (construct I) or the PEGylated 4.0-domain dimeric sTNFR-I construct (construct V). In contrast, the most modest immunological responses were seen in baboons receiving the monomeric sTNFR-I constructs. In general, when antibody responses developed, they tended to become stronger after the second and third injection.

Study 2: Efficacy study. Based on the results of the initial study, we concluded that the PEGylated 2.6-domain monomeric sTNFR-I construct was nonimmunogenic in the baboon when administered three times. To determine whether this nonimmunogenic construct was effective in blocking TNF-α-mediated injury as a dimeric 4.0-domain construct in naive animals, baboons were pretreated with either the 4.0-domain dimeric sTNFR-I construct (construct V) at 1.0 mg/kg BW, or the monomeric sTNFR-I construct (construct IV) at 1.0, 5.0, or 10 mg/kg BW (n = 3 each) 1 h before the administration of lethal quantities of E. coli. A fifth group received only placebo injections before E. coli infusions. The choice of the dimeric construct and the quantities employed were based on our laboratory’s prior studies (9, 20), which demonstrated that this dose of dimeric 4.0-domain sTNFR-I construct was protective in the baboons. The range of doses of the administered monomeric sTNFR-I construct was subsequently based on the relative neutralization capacity of two different constructs under in vitro conditions in the WEHI 164 clone 13 cell-based assay.

Survival. All three of the baboons pretreated only with placebo died or required euthanasia (for animal welfare reasons) within 5–30 h after E. coli challenge (Fig. 2). In contrast, all three of the baboons treated with the 4.0-domain dimeric sTNFR-I construct survived 14 days and were euthanized in good health at the end of the study. Survival in baboons treated with the monomeric 2.6-domain sTNFR-I construct was dose dependent. One of three baboons treated with 1.0

Table 3. Elimination half-life estimates of pharmacokinetics in baboons

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
</tr>
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<tbody>
<tr>
<td>t₁/₂ h</td>
<td>82.0 ± 32.0</td>
<td>51.2 ± 7.3</td>
<td>47.0 ± 2.4</td>
<td>40.2 ± 12.6</td>
<td>33.5 ± 2.9</td>
<td>31.1 ± 6.8</td>
</tr>
<tr>
<td>Antibody titers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Animal 1</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Animal 2</td>
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<td>0</td>
<td>0</td>
<td>400</td>
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<tr>
<td>Animal 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Dose 1 (days 0–21)

| t₁/₂ h    | 5.96 ± 0.70* | 52.0 ± 4.0 | 32.2 ± 3.7 | 47.0 ± 2.5 | 15.2 ± 5.4* | 32.0 ± 13.2 |
| Antibody titers | | | | | | |
| Animal 1 | 100 | 0 | 200 | 0 | 3,200 | 200 |
| Animal 2 | 0 | 0 | 0 | 0 | 3,200 | 0 |
| Animal 3 | 200 | 0 | 0 | 0 | 0 | 1,600 |

Dose 2 (days 21–42)

| t₁/₂ h    | 5.8 ± 0.813* | 49.1 ± 27.8 | 31.9 ± 7.03 | 42.5 ± 17.9 | 7.7 ± 6.9* | 18.5 ± 17.4 |
| Antibody titers | | | | | | |
| Animal 1 | 3,200 | 200 | 0 | 0 | 6,400 | 3,200 |
| Animal 2 | 3,200 | 0 | 0 | 0 | 1,600 | 0 |
| Animal 3 | 1,600 | 0 | 0 | 0 | 200 | 20,500 |

Half-life (t₁/₂) values are means ± SD. *P < 0.05 vs. dose 1, by paired t-test.

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mg/kg BW survived, whereas two of the three baboons treated with 5.0 mg/kg BW and all three of the baboons treated with 10 mg/kg BW of the monomeric 2.6-domain construct survived.

Hemodynamically, the placebo-treated baboons given *E. coli* developed significant hypotension and tachycardia (Fig. 3). Treatment with the dimeric sTNFR-I had no effect on the tachycardia but prevented the fall in blood pressure. Similarly, the monomeric sTNFR-I construct, when administered at 10 mg/kg BW, was also effective at preventing the hypotension. Lower doses were protective but to a lesser and dose-dependent extent.

**Plasma TNF-α immunoactivity and bioactivity.** Administration of *E. coli* to the placebo-treated baboons resulted in the rapid appearance of TNF-α immunoactivity and cytotoxicity that peaked within 90 min and declined thereafter (Fig. 4). By 4 h, all TNF-α activity had returned to baseline. In baboons treated with either the monomeric or dimeric sTNFR-I constructs, TNF-α immunoactivity was markedly prolonged, and plasma TNF-α immunoactivity remained elevated for at least 8 h. However, it appears that the majority of the TNF-α immunoactivity was bound and inactive by the sTNFR-I because the dimeric sTNFR-I construct reduced TNF bioactivity (cytotoxicity) and the monomeric construct reduced TNF-α bioactivity in a dose-dependent fashion at the higher doses (5 and 10 mg/kg BW). At 14 days, none of the surviving baboons that were administered the monomeric sTNFR-I construct (construct IV) had developed antibodies to the administered protein (data not shown).

**Other cytokines.** Treatment with both the dimeric 4.0 domain (1 mg/kg BW) and the higher doses (5 and 10 mg/kg BW) of the monomeric sTNFR-I constructs significantly attenuated both the peak IL-1β and IL-6 concentrations in the baboons, and the response to the monomeric sTNFR-I construct was dose dependent (Table 4). At 10 mg/kg BW, the monomeric construct was as effective as the 4.0-domain dimeric construct. IL-8 concentrations were not affected by the treatments (data not shown).

**Hematology responses.** *E. coli* administration in the placebo-treated baboon produced a profound leukopenia that was sustained until the animal expired. Pretreatment with the dimeric 4.0-domain sTNFR-I co-

![Fig. 2. Survival responses in *E. coli* bacteremic baboons treated with monomeric and dimeric sTNF p55 type I (sTNFR-I). Baboons (n = 3) were pretreated with either 1 mg/kg body wt (BW) of 4.0-domain dimeric sTNFR-I (construct V) or 1, 5, or 10 mg/kg BW of 2.6-domain monomeric sTNFR-I (construct IV) before the intravenous administration of live *E. coli*. Survival was evaluated over 14 days. All of the *E. coli* baboons that were untreated died, whereas none of the baboons treated with the 4.0-domain dimeric sTNFR-I expired. The 2.6-domain monomeric sTNFR-I protected in a dose-dependent fashion, with complete survival obtained at 10 mg/kg BW.](http://jap.physiology.org/)

![Fig. 3. Hemodynamic responses in *E. coli* bacteremic baboons pre-treated with escalating doses of a 2.6-domain monomeric sTNFR-I. Baboons were pretreated with either 1, 5, or 10 mg/kg BW 2.6-domain monomeric sTNFR-I (construct IV) before the intravenous administration of live *E. coli*. Hemodynamic responses were evaluated over 8 h and compared with baboons pretreated with a 4.0-domain dimeric construct (construct V) at 1.0 mg/kg BW. Both the monomeric 2.6-domain (at 5 and 10 mg/kg BW) and the dimeric 4.0-domain constructs prevented the hypotension that resulted after *E. coli* administration (P < 0.05 by two-way analysis of variance). To reduce the complexity of the graph, SE bars were deleted. BPM, beats/min.](http://jap.physiology.org/)

![Fig. 4. Plasma TNF-α immunoactivity and bioactivity in *E. coli* bacteremic baboons treated with monomeric and dimeric sTNFR-I constructs. Plasma TNF-α immunoactivity and bioactivity were determined at various times after administration of *E. coli*. Bars represent the mean ± SE of three baboons.](http://jap.physiology.org/)
shown), which confirms the accuracy of the deduced cDNA library produced an identical sequence (data not from sequencing the purified PCR product). Further- sequence was obtained either from individual clones or tive changes located in identical, without gaps, with five relatively conserva-
ies. As shown in Fig. 5, the baboon sTNFR-I domains
obtained the cDNA sequence of the baboon TNFR-I extracellular domain for comparing amino acid identi-
various human sTNFR-I constructs into baboons, we
ened the duration of neutropenia (Table 4). Similar results were seen in the surviving baboons treated with the monomeric 2.6-domain sTNFR-I construct.

Cloning of the baboon TNFR-I. To help interpret the immunogenicity data from systemic administration of various human sTNFR-I constructs into baboons, we obtained the cDNA sequence of the baboon TNFR-I extracellular domain for comparing amino acid identities. As shown in Fig. 5, the baboon sTNFR-I domains were very highly conserved in these higher primates. The cognate baboon amino acid sequence was ~97.4% identical, without gaps, with five relatively conserva-
tive changes located in domains 1, 3, and 4. This same sequence was obtained either from individual clones or from sequencing the purified PCR product. Furthermore, cDNA clones obtained from a baboon tissue cDNA library produced an identical sequence (data not shown), which confirms the accuracy of the deduced protein sequence shown. For comparison, the amino acid homology between the mouse and human TNFR-I across this same region of the protein is only ~63%.

DISCUSSION

The present study confirms that sTNFR-I constructs can be developed that are safe, have extended biological half-lives compared with native sTNFR-I, have reduced nonimmunogenicity, and can block the pathological effects of a systemic TNF-α response. In fact, we have identified one sTNFR-I construct that was non-immunogenic in the baboon when administered repeatedly over 2 mo at clinically relevant doses. In addition to identifying an optimal compound for further clinical investigation, the studies also provide important information about factors determining immunogenicity and efficacy of these constructs in vivo.

The six different constructs employed in the present study differed in three major ways: 1) the sTNFR-I was covalently bound either to a human immunoglobulin (IgG1; immunoadhesin) or to PEG, 2) the number of functional domains of the extracellular region of the sTNFR-I was either 2.6 or 4.0, and 3) the PEG was covalently linked to either monomeric or dimeric sTNFR-I. Although a random block design was not employed, there were sufficient numbers of groups to ascertain the relative contribution of each variable to the clearance and immunogenicity in the baboon.

We can speculate that the use of PEG to extend the biological half-life of the constructs was probably not responsible for the immunogenicity of the constructs, as the immunogenicity between a dimeric 4.0-domain immunoadehsin (IgG1) (construct I) and an identical dimeric PEGylated construct were similar (construct V) (Table 3). Rather, the data appear to suggest that increasing the number of functional domains of the sTNFR-I and its presence in dimeric forms were the primary contributors to immunogenicity. The most antige-
ic forms of the construct were dimers composed of 4.0-domain sTNFR-I, whereas the one construct that was nonimmunogenic was a monomeric 2.6-domain sTNFR-I. In a previous report (20), our laboratory demonstrated that reducing the number of functional domains from 4.0 to 2.6 also decreased the immunoge-
icity of a dimeric construct, suggesting that some of

<table>
<thead>
<tr>
<th>Group</th>
<th>Peak IL-6, pg/ml</th>
<th>Peak IL-1β, pg/ml</th>
<th>WBC nadir</th>
<th>WBC 8 h</th>
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<tbody>
<tr>
<td>Placebo</td>
<td>584 ± 221</td>
<td>1,368 ± 361</td>
<td>1,266 ± 120</td>
<td>3,033 ± 656</td>
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<tr>
<td>1 mg/kg</td>
<td>875 ± 366</td>
<td>1,794 ± 208</td>
<td>1,366 ± 425</td>
<td>1,266 ± 425</td>
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<td>5 mg/kg</td>
<td>364 ± 133</td>
<td>2,247 ± 816</td>
<td>1,766 ± 392</td>
<td>2,866 ± 669</td>
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<tr>
<td>10 mg/kg</td>
<td>85 ± 19</td>
<td>720 ± 117</td>
<td>1,500 ± 346</td>
<td>4,600 ± 971</td>
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<td>4.0-domain</td>
<td>23 ± 38</td>
<td>1,671 ± 311</td>
<td>1,767 ± 821</td>
<td>5,733 ± 1,027</td>
</tr>
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<td>dimeric</td>
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<tr>
<td>(1 mg/kg)</td>
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Values are means ± SE. The doses identified as 1.5, and 10 mg/kg refer to the 2.6-domain sTNFR-I monomer (construct IV). IL, interleukin; WBC, white blood cell. *P < 0.05 by one-way ANOVA and Student-Newman-Keuls post hoc testing.
the immunogenicity is directed against this region of the molecule. Similarly, in patients receiving a sTNFR-I immunoadhesin for rheumatoid arthritis, several of the antibodies that developed against the immunoadhesin recognized epitopes in the fourth functional domain (5). In a previous report, our laboratory demonstrated that removal of the fourth and part of the third domain had no effect on the construct’s neutralization capacity of TNF-α, either under in vivo or in vitro conditions (20). Similar results are reported here (Fig. 1). Crystal structure of the sTNFR-I binding to homotrimeric TNF-β confirms that the major sites of interaction between ligand and receptor are in the first and second functional domains (3).

Similarly, dimeric forms of sTNFR-I appeared to be more immunogenic as a group than monomeric forms. This reduced immunogenicity to monomeric sTNFR-I appears to be achieved with some loss of biological activity. In the in vitro studies reported here, approximately 20 times as much monomeric sTNFR-I was required to neutralize TNF-α as the dimeric construct. Similarly, in this acute, lethal model of bacteremia, 10 times as much monomeric sTNFR-I was required to protect the baboons than a comparable monomeric construct that were 50 times higher than in the initial study were also nonimmunogenic in the survival, leukocyte kinetics, plasma IL-6 and IL-1, how-ever, low doses of the monomeric sTNFR-I construct had comparable survival to that seen with baboons treated with 1 mg/kg BW of the dimeric sTNFR-I construct. Similarly, the capacity to reduce the hemodynamic changes, the proinflammatory cytokine levels (IL-1 and IL-6), and the hematological responses were similar between baboons treated with the dimeric sTNFR-I construct at 1.0 mg/kg BW and the monomeric sTNFR-I construct at 5.0 and 10 mg/kg BW. Doses of the monomeric sTNFR-I construct that were 50 times higher than in the initial study were also nonimmunogenic in the surviving E. coli-treated baboons.

The plasma TNF-α response was surprisingly different among the treatment groups (Fig. 4). Although both constructs significantly prolonged the appearance of immunological TNF-α in the circulation, baboons treated with the dimeric 4.0-domain sTNFR-I construct had essentially no detectable free TNF-α bioactivity. In contrast, treatment with the monomeric 2.6-domain sTNFR-I construct appeared to reduce the free TNF-α activity in a dose-dependent fashion, albeit not completely. Interestingly, low doses of the monomeric sTNFR-I construct (1 mg/kg BW) appeared not to neutralize TNF-α bioactivity, and the plasma IL-1β and IL-6 responses were also unaffected. At these low concentrations, the monomeric sTNFR-I construct may have prolonged the life and bioactivity of the TNF-α. All of the physiological parameters evaluated (survival, leukocyte kinetics, plasma IL-6 and IL-1), however, are consistent with a reduction in TNF-α pathology in vivo associated with the neutralization of TNF-α bioactivity as the dose of monomeric sTNFR-I was increased.

It should also be noted that this differential dose requirement between the dimeric 4.0-domain and monomeric 2.6-domain sTNFR-I constructs in the baboon...
IMMUNOGENICITY AND EFFICACY OF STNFR-I CONSTRUCTS

has not been seen in rodent models of chronic inflammation. Bendele and colleagues (4, 16) have observed that, in a rat model of adjuvant arthritis, 4.0-domain sTNFR-I immunoadhesins and dimeric and monomeric PEGylated sTNFR-I constructs were equally effective in blocking the disease progression, and monomeric 2.6-domain sTNFR-I constructs were nonimmunogenic.

The question that naturally arises is whether these findings in primates can be extended to humans. We did not evaluate immunogenicity to repeated administration of the monomeric sTNFR-I construct at doses equivalent in neutralization activity to the dimeric construct. In contrast, we chose to evaluate immunogenicity at doses that are likely to be used in humans with rheumatoid arthritis (18). Nevertheless, evaluating immunogenicity with human proteins in primates is still limited by species specificity. To that end, we cloned the baboon sTNFR-I and compared the predicted amino acid sequence from the transcribed region of the TNFR-I cDNA of humans. The transcribed region for the extracellular domain of the baboon TNFR-I differed by only 5 amino acids out of 193 (97% homology). Because the human 2.6-domain monomeric sTNFR-I was not immunogenic in the baboon after three injections, even with two amino acid deviations, we believe that this strong homology argues that the same construct will also have reduced immunogenicity in humans. We cannot, however, conclude that the 4.0-domain constructs, which have greater immunogenicity in the baboon, will be similarly immunogenic in humans. However, significant immunogenicity has been seen with the 4.0-domain sTNFR-I immunoadhesins administered to patients with rheumatoid arthritis and multiple sclerosis (5). Of course, future clinical trials are required to determine whether this monomeric 2.6-domain construct will be suitable for the treatment of human disease.

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REFERENCES


