

Pharmacokinetics, immunogenicity, and efficacy of dimeric TNFR binding proteins in healthy and bacteremic baboon

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Solorzano, Carmen C., Atsushi Kaibara, Phillip J. Hess, Paul D. Edwards, Riadh Ksontini, Amer Abouhamze, Sherry McDaniel, Janet Frazier, Deborah Trujillo, Gary Kieft, James Seely, Tadahiko Kohno, Mary Ellen Cosenza, Michael Clare-Salzler, Sally L. D. MacKay, Steven W. Martin, Lyle L. Moldawer, and Carl K. Edwards III. Pharmacokinetics, immunogenicity, and efficacy of dimeric TNFR binding proteins in healthy and bacteremic baboon. *J. Appl. Physiol.* 84(3): 1119–1130, 1998.— Immunogenicity, pharmacokinetics, and therapeutic efficacy of three novel dimeric soluble tumor necrosis factor (TNF)-receptor I constructs [TNF-binding protein (bp)] were evaluated in 28 baboons, 12 of which were healthy and 16 were challenged with a lethal *Escherichia coli* bacteremia. The three constructs differed only in the number of extracellular domains of the TNF receptor I and were dimerized with polyethylene glycol. Although all three constructs had generally similar pharmacokinetics when administered to a naive animal, they differed quantitatively in their immunogenicity. Antibodies were detected more frequently, and titers were significantly higher ($P < 0.05$) in both healthy and septic baboons that received the 4.0-domain TNF-bp construct, compared with animals receiving the 2.6-domain construct. When the TNF-bp constructs were administered a second time (21 days later), the half-lives of the three constructs were significantly shorter in animals that had an antibody response after the first injection. In contrast, all three TNF-bp constructs were equally effective at improving outcome, blocking a systemic TNF- α response, and attenuating the cytokine responses when administered at a dose of 1.0 mg/kg body wt 1 h before a lethal *E. coli* infusion. The findings suggest that immunogenicity of TNF-bp constructs can be altered by changing the number of functional domains, without affecting their capacity to neutralize TNF- α and to abrogate TNF-mediated pathology.

tumor necrosis factor receptor; sepsis; baboons; antibodies

THERE IS GENERAL AGREEMENT that exaggerated production of tumor necrosis factor- α (TNF- α) contributes to the pathogenic response following a variety of acute or chronic inflammatory processes (3, 6, 17, 25). Sepsis, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and reperfusion injury are some inflammatory diseases in which TNF- α has been implicated. Clinical studies are currently underway examining the effectiveness of TNF- α blockade in patients with sepsis syndrome, rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis.

Efforts to block endogenous TNF- α production have focused primarily on either small molecules that inhibit TNF- α production or processing or proteins that block TNF- α binding to its receptors. The former include inhibitors of macrophage activation and TNF- α transcription, as well as TNF- α processing (13, 19, 23). Protein-based approaches have included monoclonal antibodies, soluble TNF-receptor (TNFR) constructs, and TNFR immunoadhesins (1, 5, 14, 26, 28). Although protein-based therapies have proven to be effective inhibitors of TNF activity, their widespread use has been limited by their cost of production, immunogenicity, and limited biological half-life.

In 1992, we reported (28) that the infusion of the extracellular domain (soluble form) of the TNF receptor I (p55 or TNFR I) could bind TNF- α in vivo and attenuate the inflammatory response to a lethal bacterial challenge. However, the half-life of the monomeric, extracellular domain of the p55 receptor was short (<2 h), and the extracellular TNFR I-TNF- α plasma complex was unstable, resulting in the release of bioactive TNF- α under in vitro conditions. The biological half-life of the extracellular TNFR I and capacity to neutralize homotrimeric TNF- α could be markedly improved by creating a TNFR I construct covalently linked to polyethylene glycol (5). When baboons were pretreated with these constructs, the subsequent inflammatory response to *Escherichia coli* bacteremia was significantly attenuated and the plasma appearance of bioactive TNF- α abrogated (5). In endotoxemic shock, concanavalin-A-induced hepatitis, and visceral ischemia-reperfusion, these TNF binding proteins (TNF-bp) constructs have proven effective at reducing organ injury (21, 23, 30).

The present study was undertaken to examine whether structural modifications of the pegylated dimeric TNFR I could alter the plasma half-life and immunogenicity of the construct while still retaining its biological efficacy. Three constructs were evaluated that differed only in the number of functional domains of the TNFR I (4.0, 3.0, 2.6). The results demonstrate that the immunogenicity and biological half-life of these constructs can be altered by structural modifications, without adversely affecting the construct's ability to block TNF- α mediated responses in a *Papio* model of *E. coli* lethality.

MATERIALS AND METHODS

Animals. Twenty-eight young adult male and female baboons (*Papio anubis*, 6–11 kg) were purchased from Biomedical Research Foundation (San Antonio, 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 they were in good health and had no transmissible diseases. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Florida before initiation of these studies. The laboratory adheres to the Guiding Principles of Laboratory Animal Care, as promulgated by the American Physiological Society.

TNF-bp constructs. Three TNF-receptor-binding protein constructs were evaluated. The three constructs were composed of varying regions of the extracellular domain of the human TNFR I, with a single amino acid substitution required for pegylation. The 2.6 domain construct contained the amino acid sequence from Met⁰ to Leu¹⁰⁸, whereas the 3.0 domain contained the sequence from Met⁰ to Thr¹²⁷, and the 4.0 domain contained the sequence from Met⁰ to Asn¹⁶¹. Figure 1 shows the three-dimensional crystal structures of the three soluble TNFR I constructs, as determined by X-ray crystallography and computer modeling (15).

The cloning and expression of the three human TNF-bp truncated forms were performed by polymerase chain reaction (PCR) amplification by using a cloned human TNFR I template and primers specific for the three constructs. PCR was run for 25 cycles; each cycle consisted of 94°C for denaturation, 15 s at 60°C for annealing, and 1 min at 72°C for elongation (Perkin-Elmer Cetus model 2400 thermocycler, Norwalk, CT), or similar conditions. The PCR product was purified by using a QIAquick PCR purification kit (Qiagen, Chatsworth, CA). The purified PCR product was digested with restriction enzymes, then gel was purified by using the QIAquick gel extraction kit. Gel-purified PCR product was ligated into pAMG11 and transformed into the *E. coli* cell line FM15.

Expressed protein was purified by ion-exchange chromatography and dimerized with sulfone-activated polyethylene glycol (PEG-20,000-*bis*-vinyl sulfone) by using the method described by Seely et al. (22). Proteins were reduced before the attachment of the polyethylene glycol with 4 mol dithiothreitol per 1 mol of protein at 5–6°C. All reactions were performed in the presence of 30% glycol.

The three constructs were obtained from Amgen (Boulder, CO). Endotoxin concentrations of all preparations were found to be <0.2 endotoxin units/mg of protein.

In vitro studies. To compare the relative capacity of this dimeric construct to neutralize homotrimeric TNF- α vs. a monomeric 4.0-domain TNF-bp, baboon plasma was spiked with 35,000 pg/ml of recombinant human TNF- α . TNF- α -spiked baboon plasma was incubated with increasing quantities (from 100 pg/ml to 100 μ g/ml) of either dimeric or monomeric 4.0-domain TNF-bp constructs for 30 min before being applied to murine WEHI 164 clone 13 fibroblasts. WEHI 164 fibroblasts were cultured in 96-well microtiter plates to near confluency in RPMI 1640 media containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Plasma sample (20 μ l) coincubated with the TNF-bp constructs and diluted 1:5 was added to each well, and cells were cultured with 1.0 μ g/ml actinomycin D overnight at 37°C. For the last 4 h, cells were incubated in the presence of 6 mg/ml of the vital chromogen. At the end of the incubation, medium was removed, and the cells were lysed with isopropanol and sterile water. Cell viability was

determined spectrophotometrically at 570/690 nm. Data are presented as the percent cell survival in the absence of any TNF- α .

Study protocol. The study was divided in two phases. *Phase I* of the study was aimed at determining the pharmacokinetics and immunogenicity of the different constructs in the healthy baboon. Twelve baboons were randomly assigned to three groups. While anesthetized, each group received 0.2 mg/kg body wt of either the 2.6-domain, 3.0-domain, or 4.0-domain TNF-bp. One baboon from each group was studied simultaneously during each session. After 21 days, the animals received a second intravenous injection of the same protein and were studied for an additional 21 days. Blood samples were collected for immunogenicity and pharmacokinetic analyses throughout both 21-day study periods.

Phase II of the study was aimed at evaluating efficacy of these preparations in a well-established model of TNF- α -mediated lethality (11, 26, 28). Lethal *E. coli* bacteremia was induced in 16 animals by administration of $5\text{--}10 \times 10^{10}$ colony-forming units/kg body wt of live *E. coli*. The animals were randomly assigned to one of four treatment groups. A placebo group was compared with baboons pretreated intravenously with either the 2.6-, 3.0-, or 4.0-domain TNF-bp administered at 1 mg/kg body wt.

Detailed study designs. In both phases of the study, after an overnight fast, animals were anesthetized with ketamine (10 mg/kg im), and the cephalic vein was percutaneously cannulated. Anesthesia was maintained by the initial administration of up to 35 mg/kg pentobarbital sodium followed by repeated injections of ~3–5 mg/kg of pentobarbital sodium, as required. The upper airway was secured by placement of a cuffed endotracheal tube, and the animals maintained spontaneous respiration. A catheter was placed percutaneously into the femoral artery, which permitted repeated systemic arterial blood sampling as well as continuous monitoring of heart rate and mean arterial blood pressure via a Datascope 2000 (San Antonio, TX) cardiac monitor. Core temperature was monitored via a rectal probe. An indwelling urinary catheter (Foley) was placed to allow urine collection and to monitor urine output and creatinine clearance. Hemodynamic parameters were monitored every 15 min. All animals received 0.9% sodium chloride (3 ml \cdot kg⁻¹ \cdot h⁻¹) as maintenance intravenous fluid delivered continuously by an infusion pump. Arterial blood samples were collected at intervals, anticoagulated with EDTA or heparin, and cooled on ice immediately after drawing. The plasma fraction was separated by centrifugation at 1,300 *g* at 4°C and stored at -70°C until assayed.

In *phase II* of the study, animals received additional fluid (10 ml/kg every 15 min) if two of the following physiological criteria were met: 1) mean arterial pressure dropped by >30%, 2) heart rate increased by >30%, and 3) urine output dropped to <1 ml \cdot kg⁻¹ \cdot h⁻¹. After baseline blood sampling and a waiting period of at least 1 h to allow equilibration, infusion of proteins was started.

In *phase I* of the study, recombinant proteins were infused as a 30-s bolus via the cephalic vein, and animals were observed for a period of 8 h, after which time all catheters were removed, and the animals were returned to their cages for 21 days. Blood samples were collected at -1 h, 0 h, 2 min, and at hourly intervals for the first 8 h. After being returned to their cages, the animals were briefly anesthetized with ketamine (10 mg/kg im) on days 1, 2, 3, 5, 8, 11, 16, and 21, and 10-ml venous blood samples were obtained. On day 21, the animals were reanesthetized, received a second injection of the same protein as administered on day 0, and the entire procedure was repeated for an additional 21 days, at which time the animals were euthanized.

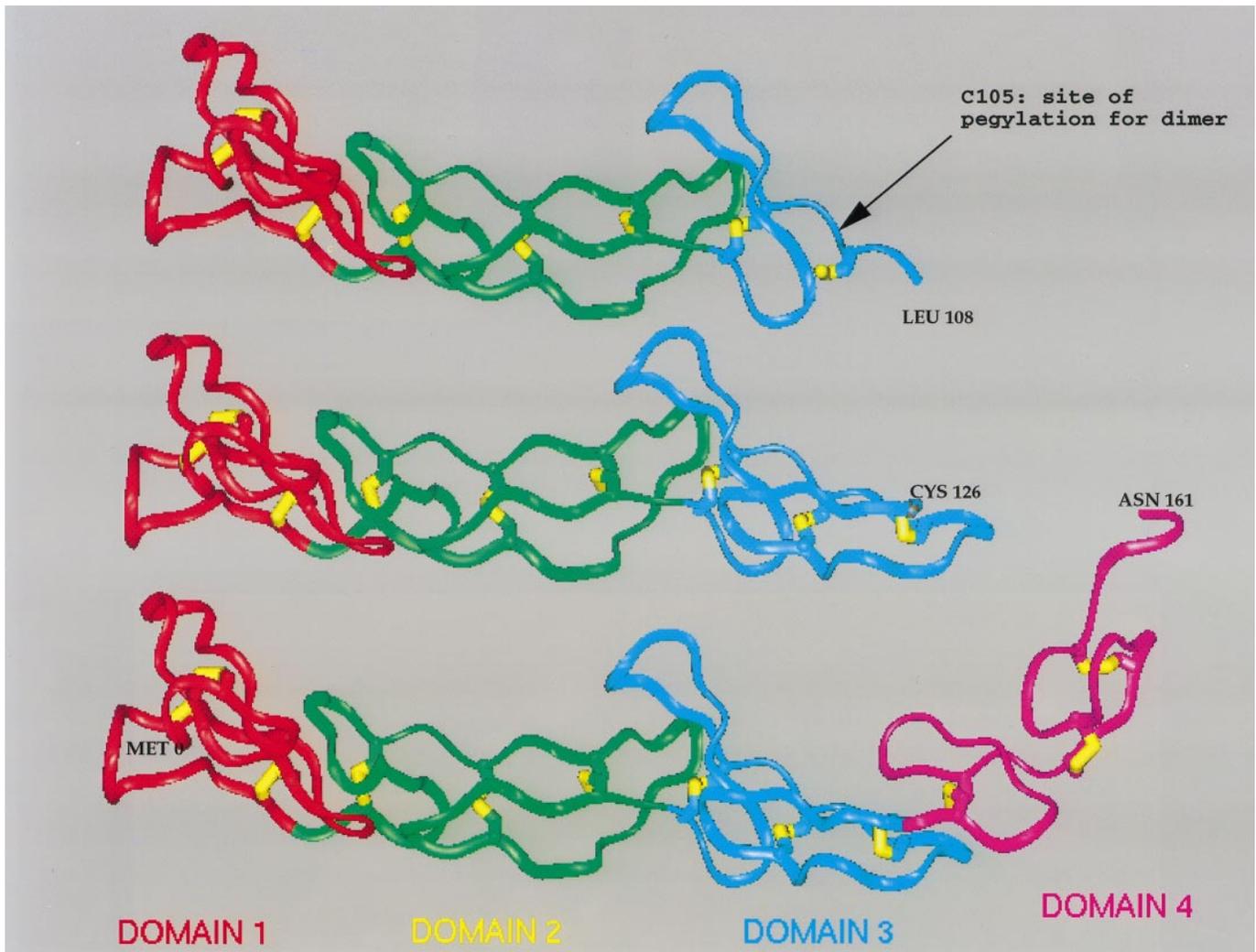


Fig. 1. Predicted crystal structure of the 3 tumor necrosis factor (TNF)-receptor (TNFR) constructs. Predicted 3-dimensional structures of the 3 TNFR constructs were obtained from X-ray crystallography and computer modeling. TNFR constructs differed in no. of functional domains, as identified here. TNFR constructs were homodimerized, as described in MATERIALS AND METHODS, with polyethylene glycol at a site in 3rd domain (amino group of AA¹⁰⁵).

In *phase II* of the study, 1 h before the infusion of *E. coli*, four animals were randomly assigned to receive either placebo or one of the three constructs. Animals were observed for a period of 8 h, after which time all catheters were removed, the animals were returned to their cages, and subsequent survival to the lethal bacteremia was observed over the next 21 days. Because of animal welfare concerns and the desire to reduce suffering in the baboons, the animals were monitored every 2–4 h by a clinical veterinarian blinded to the treatment of the animals. If the animals were judged to be moribund, suffering excessive discomfort despite appropriate analgesia, and likely to expire within the next few hours, the baboons were euthanized. The criteria used to judge impending death and excessive discomfort (as defined by the Institutional Animal Care and Use Committee) were 1) failure to maintain the sitting or upright position over the previous 12 h, 2) failure to take any food or water within the previous 12 h, 3) uncontrollable bleeding from catheter sites, or 4) unresponsiveness to external stimuli. These criteria have been used previously instead of allowing the animals to die spontaneously (29).

Venous blood samples were collected at –1, 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 24, and 48 h, and on *days 3, 5, 8, 11, 16, and 21*. At 21 days, surviving animals were euthanized.

Analytic assays. Plasma TNF- α activity was determined by both enzyme-linked immunosorbent assay (ELISA) and by plasma-based bioassay. The TNF- α sandwich ELISA employs a monoclonal antibody as the capture and a polyclonal rabbit anti-TNF- α antiserum as the secondary antibody. The ELISA can recognize both free TNF- α and TNF- α bound to either of its soluble TNF receptors (29), although the affinity for TNF- α bound to its shed receptor is reduced. TNF- α bioactivity was assessed by using the WEHI 164 clone 13 cytotoxicity assay (28), which detects only free bioactive protein. In addition, the capacity of plasma from baboons that received each of the three TNF-bp constructs to neutralize excess TNF- α was determined. In this case, serial dilutions (from 1:10 to 1:20,000) of plasma from baboons treated with either the 4.0-, 3.0-, or 2.6-domain TNF-bp construct obtained 90 min after *E. coli* administration (peak endogenous TNF- α production) was coincubated with 310 pg/ml of added recombinant human TNF- α (95% killing of WEHI cells).

Interleukin (IL)-1 β , IL-6, and IL-8 concentrations were measured by ELISA as previously described (11). The plasma concentrations of the TNF-bp constructs were determined by sandwich ELISA with the use of monoclonal and polyclonal antibodies raised against the TNFR I component of the construct. The recombinant proteins were each used as their respective standards for the ELISA, and the sensitivity of the assay was 32 pg/ml.

The presence of *Papio* antibodies to the administered recombinant proteins was determined by sandwich ELISA. Very briefly, the TNF-bp constructs were coated onto ELISA plates (1 μ g/ml), and diluted (1:50 to 1:100,000) baboon plasma (100 μ l) was added. After the samples were washed, a horseradish peroxidase-conjugated protein A was added (0.5 μ g/ml), and the assays were visualized with 3,3',5,5'-tetramethylbenzidine.

To determine whether the antibodies were neutralizing, an L-929 neutralizing antibody assay was performed (20). Plasma samples from selected baboons were incubated with the respective TNF-bp construct and, subsequently, with recombinant human TNF- α . The solutions were then added to L-929 cells (5×10^4 /ml) in 96-well microtiter plates with complete RPMI 1640 medium containing 10% fetal calf serum, 1.5 mM L-glutamine, 220 U/ml penicillin, 220 μ g/ml streptomycin, and 1.2 mg/ml of actinomycin D. Cell viability was determined 19–21 h later with 0.2% crystal violet. Controls in the bioassay included serial dilutions (2,500 ng/ml to 0.25 μ g/ml) of recombinant human TNF- α as a cytotoxic control, 10 μ g/ml of a neutralizing monoclonal antibody against human TNF- α (R&D Systems, Minneapolis, MN) as a positive control, 0% (TNF-bp plus human TNF- α) and 100% (human TNF- α) neutralization controls, a prestudy plasma spiked with neutralizing monoclonal antibody as a serum-effect control, and serial dilutions of the TNF-bp construct. Percent neutralization was calculated from the ratio of the killing with plasma sample minus the preplasma spike divided by the neutralizing antibody control.

To determine whether the antibodies produced in response to the TNF-bp constructs were cytotoxic, by virtue of their cross-linking of the TNFR I on the cell surface, the human epidermoid carcinoma cell line ME-180 was employed. Replicate points of a predose plasma diluted 1:12 were positionally paired in 96-well microtiter plates, with replicate points from posttreatment plasma diluted 1:12 with exponentially growing cells in RPMI 1640 medium containing 10% fetal calf serum, 1.5 mM L-glutamine, 220 U/ml penicillin, 220 μ g/ml streptomycin, and 1.2 mg/ml of actinomycin D. Cell viability was determined 19–21 h later with 0.2% crystal violet. Controls for the assay on a per plate basis consist of serial dilutions of TNF- α as a cytotoxic control, purified normal goat immunoglobulin G (IgG) as a negative control, and affinity purified goat anti-human TNF-bp IgG as a positive control. The predose and postdose plasma results were compared to detect a difference that is both significant and indicative of a cytotoxic effect.

Pharmacokinetics. The parameters defining the plasma characteristics were determined by noncompartmental analysis using WinNonLin (version 1.1, Scientific Consulting, Lexington, KY). Endogenous levels of baboon soluble TNFR I were detected in predose samples on *day 0*; mean baseline value was 0.98 ± 0.26 (SD) ng/ml ($n = 24$), which indicates cross-reactivity between endogenous soluble TNFR I and the assay. Therefore, baseline correction of the data was performed for each animal by subtracting its own predose level of soluble TNFR I from all plasma TNF-bp levels recorded after treatment with the test material. In placebo-treated bacteremic animals, the endogenous levels of soluble TNFR I in-

creased over 20-fold during the first 2 days and then declined to predose values after 10 days. Therefore, background subtraction of data in the TNF-bp-treated bacteremic animals was performed by using the mean profile obtained from the placebo-treated animals (data not shown). The maximum observed baseline-corrected plasma concentration and sample time at which this occurred were determined from the data. Area under the baseline-corrected plasma-concentration time curve and area under the first-moment curve to infinity were estimated by combination of linear interpolation up to the time at maximum concentration, followed by the log trapezoidal rule during the declining portion of the curve. Extrapolation of the plasma concentration from the last time point to infinity was determined by log-linear regression analysis. Additional noncompartmental parameters determined were steady-state volume of distribution and terminal half-life.

To investigate whether differences existed in the initial half-life, the parameters defining the plasma characteristics were also determined by compartmental analysis with the use of SAAM II (version 1.1, SAAM Institute, Seattle, WA). A two-compartment disposition model incorporating first-order elimination provided a good description of the data. The model was optimized to individual profiles by using a constant covariance (10%) relative weighting scheme based on the data, the Rosenbrock integrator, and a convergence criterion of 1×10^{-4} .

Hematologic 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,

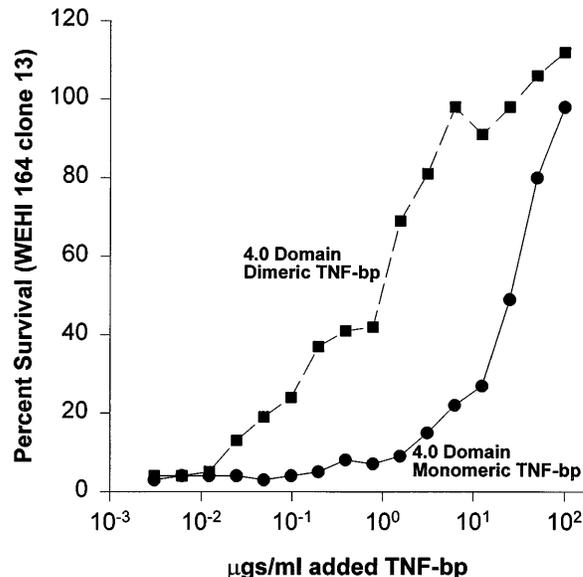


Fig. 2. Neutralization capacity of dimeric and monomeric forms of TNF-binding proteins (bp). WEHI 164 clone 13 cells were cocultured with baboon plasma containing 35,000 pg of recombinant human TNF- α and increasing quantities of monomeric and dimeric 4.0-domain TNF-bp. Cell viability was determined 24 h later, and data are presented as %cells surviving (vs. WEHI 164 clone cells grown in absence of TNF- α). Quantity of monomeric TNF-bp required to neutralize 50% of TNF- α -mediated killing was ~20-fold greater than for dimeric TNF-bp. Ongoing binding affinity and association and disassociation rates for the dimeric and monomeric TNF-bp analogs are currently being assessed by BIAcore analyses. Actual binding affinities of monomeric TNF-bp proteins do not appear different from dimeric TNF-bp proteins in initial studies (data not shown).

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. Serum and urine creatinine were determined spectrophotometrically by using an automated procedure at the University of Florida College of Veterinary Medicine.

Histopathological analyses. Because of the concern of renal vacuolization secondary to repeated polyethylene glycol administration, histological examinations of the kidneys were performed. At time of necropsy, secondary to either euthanasia for animal welfare concerns or at the completion of the study, kidneys were removed and fixed immediately in buffered Formalin. Specimens were not obtained from the occasional animal that died spontaneously from sepsis before euthanasia. Formalin-fixed specimens were sectioned at 5 μ m, embedded in paraffin, and stained with hematoxylin and eosin. Blinded samples were scored (scale 0–4; from absence of to widespread and severe) by a veterinary pathologist for degree of vacuolization, medullary and cortical necrosis, presence of inflammatory infiltrates, and degree of thrombin or fibrin deposition.

Statistical analyses. All values are expressed as means \pm SE unless otherwise stated. Differences among the groups

Table 1. *Pharmacokinetics and volumes of distribution*

	Initial Half-Life, h	Terminal Half-Life, h	Clearance, ml \cdot h ⁻¹ \cdot kg body wt ⁻¹	V _{ss} , ml/kg
<i>Phase I: healthy baboons, first injection</i>				
2.6 Domain	8.06 \pm 1.29	29.17 \pm 4.57	3.71 \pm 0.27	96.9 \pm 8.3
3.0 Domain	7.49 \pm 2.08	28.03 \pm 1.87	3.77 \pm 0.38	100.9 \pm 6.2
4.0 Domain	10.62 \pm 2.94	34.23 \pm 0.62	2.54 \pm 0.33	103.9 \pm 10.8
<i>Phase I: healthy baboons, second injection</i>				
2.6 Domain	6.03 \pm 0.61	26.53 \pm 6.80	3.79 \pm 0.60	70.3 \pm 16.0
3.0 Domain	6.77 \pm 1.34	16.17 \pm 6.10	4.78 \pm 0.84	100.9 \pm 11.9
4.0 Domain	14.09 \pm 0.70	15.92 \pm 1.69*	3.78 \pm 0.72	89.6 \pm 12.6
<i>Phase II: Escherichia coli bacteremic baboons</i>				
2.6 Domain	3.09 \pm 0.60†	22.53 \pm 1.63	3.75 \pm 0.21	88.0 \pm 10.2
3.0 Domain	3.26 \pm 0.77	23.78 \pm 1.80	4.74 \pm 0.60	135.7 \pm 13.6
4.0 Domain	1.89 \pm 0.66†	28.84 \pm 3.09	2.82 \pm 0.77	86.9 \pm 10.2

Values are means \pm SD; $n = 4$ baboons/group. V_{ss}, volume of distribution at steady state. * $P < 0.05$ vs. 1st injection, by paired Student's t -test; † $P < 0.05$ vs. 1st injection in healthy baboons, by 2-way analysis of variance (ANOVA).

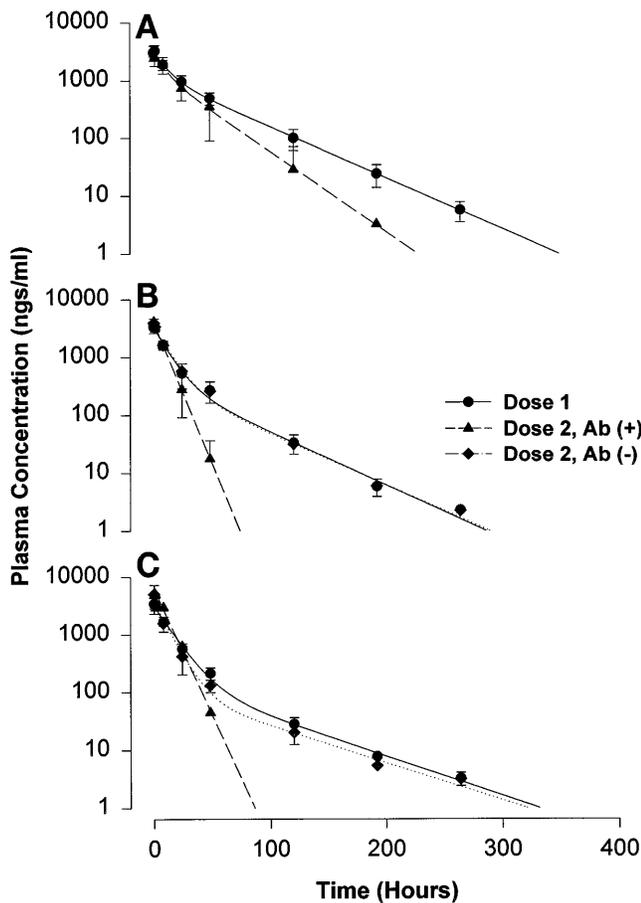


Fig. 3. Plasma TNF-bp concentrations in healthy baboons treated twice with either the 4.0 (A), 3.0 (B), or 2.6-domain (C) constructs. Baboons were anesthetized, and 0.2 mg/kg body wt of the 3 constructs was intravenously infused. Blood samples were obtained at intervals described over 21 days, and volumes of distribution and clearance rates were determined by using a noncompartmental analysis. Disappearance curves presented are from 1st 21 days in all baboons (dose 1; ●) and 2 curves are provided for 2nd 21-day period, one for animals that developed an antibody response [dose 2, antibody (Ab) (+); ▲] and one for animals that did not develop it [dose 2, Ab (-); ◆].

were analyzed by one- and two- way analyses of variance. In some cases, a mixed-effect model was employed. For those samples that did not achieve normality, a Kruskal-Wallis nonparametric analysis of variance was performed. Post hoc comparisons were performed by using Dunnett's test. Differences in survival and frequency of antibody detection were determined by χ^2 analysis or Fisher's exact test. In all cases, significance was determined at the 95% confidence interval.

RESULTS

In vitro studies. To determine the relative capacity of dimeric and monomeric forms of TNF-bp, baboon plasma was coincubated with shock levels of recombinant human TNF- α (35,000 pg/ml) and increasing quantities of monomeric and dimeric 4.0-domain TNF-bp. The quantities of TNF-bp required to neutralize TNF- α activity are presented in Fig. 2. On a weight basis, the dimeric form of TNF-bp was \sim 20-fold more effective as the monomeric form in neutralizing 50% of the TNF- α activity. The approximate mean effective concentration for dimeric TNF-bp was 1 μ g/ml, whereas the mean effective concentration for monomeric TNF-bp was 20 μ g/ml.

Phase I physiological analyses. Administration of the three TNF-bp constructs to the healthy naive primates was without any acute hemodynamic or hematologic effect as evaluated over the initial 8 h of constant monitoring. Mean arterial blood pressure, heart rate, core temperature, urine output, and urine creatinine clearance were unaffected by treatment with any of the three constructs (data not shown). Furthermore, on day 21, when the animals were reinjected with additional quantities of the same constructs as administered on day 0, no adverse physiological responses were noted. Thus the three constructs appeared to be safe in the anesthetized baboon.

Phase I pharmacokinetic analyses. The parameters describing the plasma kinetics of the three different dimeric forms of the TNF-bp are shown in Fig. 3 and Table 1. The initial half-life, final half-life, clearance,

Table 2. Peak antibody responses

	Phase I: Healthy Baboons						Phase II: Septic Baboons		
	First 21-day period			Second 21-day period			21 Days		
	Median	25%	75%	Median	25%	75%	Median	25%	75%
4.0 Domain	3.20	3.20	3.20	3.95	3.50	4.40	3.96	3.66	4.56
3.0 Domain	1.60	0.00	3.65	3.50	1.30	4.75	3.35	2.90	4.10
2.6 Domain	0.00*	0.00	1.75	1.45	0.00	3.50	3.20	1.60	3.50

Values are means \pm SE; $n = 4$ baboons/group. Logarithmic scale was used for responses (inverse dilution of plasma necessary to produce half-maximal absorbance on a sandwich enzyme-linked immunosorbent assay; see *Experimental Methods*). * $P = 0.056$, by Kruskal-Wallis 2-way ANOVA (log-transformed values failed tests of normality).

and volume of distribution at steady state (V_{ss}) for the three constructs did not differ significantly among the three groups, although the elimination half-life tended to be longer and the clearance to be lower for the 4.0 domain construct.

The major change in the pharmacokinetics of these compounds after the second dose was an effect on the terminal half-life, which tended to be shorter for all

compounds (Table 1). The decrease in terminal half-life was more pronounced for the 4.0-domain construct in which it decreased by 54% ($P < 0.01$), compared with a 43% [$P =$ not significant (NS)] and 9% ($P =$ NS) for the 3.0- and 2.6-domain constructs, respectively. In addition, clearance for all compounds tended to be greater after the second dose but was not statistically significant. No significant change was observed in the V_{ss} or initial half-life between the first and second injection for any of the TNF-bp constructs.

Phase I antibody responses. All of the preparations were immunogenic in the baboons (Table 2, Fig. 4). Antibody responses generally developed around the eighth day after administration of the constructs and were present through the 21-day study period. Furthermore, antibody responses tended to become stronger after the second injection of the protein constructs.

On day 21, all four of the baboons receiving the 4.0-domain construct had developed antibodies, whereas two of four of the animals receiving the 3.0-domain construct and one of the four baboons receiving the 2.6-domain construct developed antibodies after the first injection. By Kruskal-Wallis analysis of variance, the magnitude of the antibody response (log transformed) was significantly different among the three groups as a function of time ($P < 0.05$). Post hoc analysis suggested that the significant difference in antibody responses was principally between animals receiving the 4.0- and 2.6-domain constructs, with intermediate (and nonsignificant) responses from the animals treated with the 3.0-domain construct. At the end of the study (day 42), only three animals had not developed antibodies: two animals receiving the 2.6-domain construct and one animal receiving the 3.0-domain construct.

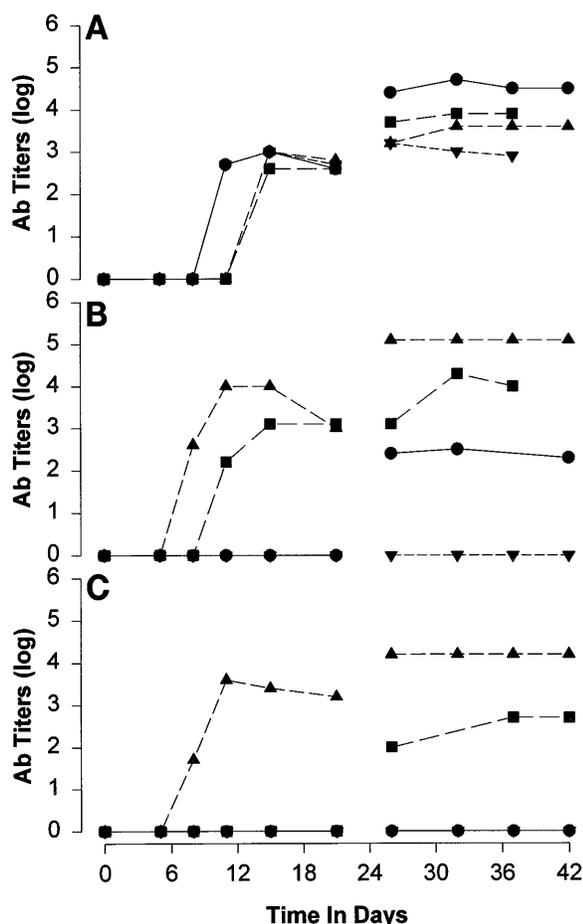


Fig. 4. Appearance of antibodies in blood after administration of 3 constructs: 4.0 (A), 3.0 (B), and 2.6 domain (C). Baboons were anesthetized, and 0.2 mg/kg body wt of 3 constructs was intravenously infused. Blood samples were obtained at intervals described over 21 days and antibodies determined as in MATERIALS AND METHODS. Antibodies were generally detected within 8 days of 1st injection and remained elevated over remaining 21-day period. Antibody responses were higher after 2nd injection. Each symbol represents a different baboon. Table 2 provides median levels and ranges for antibody responses among the 3 groups.

Table 3. Cytotoxicity and neutralizing capacity of plasma from selected baboons treated with TNF-bp constructs in the L-929 and ME-180 murine cell lines

Group	Day of Sample	L-929 Neutralization (1:240 to 1:2.4 $\times 10^6$)	ME-180 Cytotoxicity (1:120)
4.0 Domain	16	–	–
	33	–	+/-
	36	–	–
3.0 Domain	11	–	–
	33	–	–
2.6 Domain	33	–	–

TNF-bp, tumor necrosis factor-binding proteins. – Indicates absence of either neutralization or cytotoxicity activity, whereas +/- indicates an indeterminate activity.

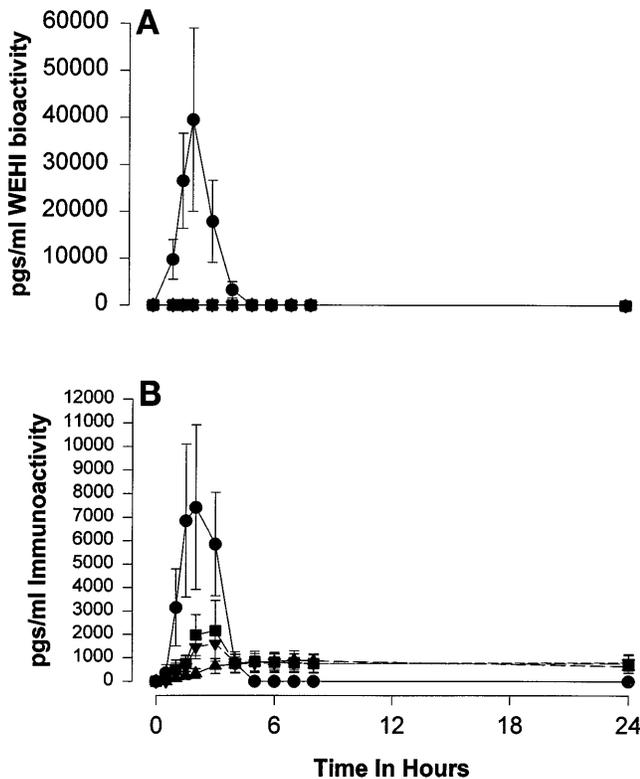


Fig. 5. Plasma TNF- α bioactivity (A) and immunoactivity (B) in *Escherichia coli*-shock baboons treated with either placebo or 1 of 3 TNF-bp constructs. Baboons were pretreated with 1.0 mg/kg body wt of either 4.0, 3.0, or 2.6-domain TNF-bp or with placebo, 1 h before administration of live *E. coli*. Blood samples were then obtained at intervals, and TNF- α bioactivity was determined by WEHI 164 clone 13 cytotoxicity, whereas TNF- α immunoactivity was determined by enzyme-linked immunosorbent assay. Pretreatment with either of 3 constructs completely eliminated free TNF- α bioactivity while extending appearance of TNF- α immunoactivity. ●, Placebo-treated animals; ▲, 4.0-domain-pretreated animals; ■, 3.0-domain-pretreated animals; and ▼, 2.6-domain-pretreated animals.

In those animals that had developed an IgG antibody response by *day 21*, the final half-lives of the TNF-bp constructs were significantly ($P < 0.001$) shorter after the second administration (Fig. 3). When the changes

in the terminal half-life between first and second injection were compared between antibody-positive (reduced by 16.1 ± 2.4 h) and -negative animals (reduced by 3.7 ± 5.8 h), the reduction in terminal half-life was significantly greater ($P < 0.05$) in antibody-positive animals. Thus it appears that the effect of antibodies was most evident during the terminal phase when the TNF-bp concentrations were < 500 ng/ml.

The antibodies that were detected in the plasma of the baboons were evaluated in a selected number of animals for direct cytotoxicity in the ME-180 cell line and neutralizing capacity in an L-929 fibroblast cell line cytotoxicity assay (Table 3). No cytotoxicity or neutralization was seen with antibodies generated to any of the three constructs.

Histologically, there was no evidence of vacuolization of the tubular epithelium (data not shown) in any of the baboons treated with the pegylated TNF-bp constructs. There was minimal subacute inflammation and mineralization in a few of the samples, but this was not considered to be related to the treatments.

Phase II efficacy study. To determine whether the three TNF-bp constructs were similarly effective in blocking TNF- α -mediated injury in naive animals, baboons were pretreated with one of the three constructs (1.0 mg/kg body wt) 1 h before the administration of lethal quantities of *E. coli*.

Phase II pharmacokinetics and immunogenicity. One hour before *E. coli* bacteremic challenge, animals were dosed intravenously with one of the three TNF-bp constructs (1 mg/kg body wt). The initial and terminal half-lives of all TNF-bp constructs tended to be shorter in bacteremic than in normal baboons (Table 1). The initial half-lives of the 2.6-domain and 4.0-domain TNF-bp were significantly ($P < 0.05$) shorter in bacteremic animals, whereas the initial half-life of the 3.0-domain TNF-bp did not reach statistical significance ($P = 0.1$). No consistent differences in the terminal half-life, plasma clearance, or V_{ss} were observed between normal and bacteremic animals.

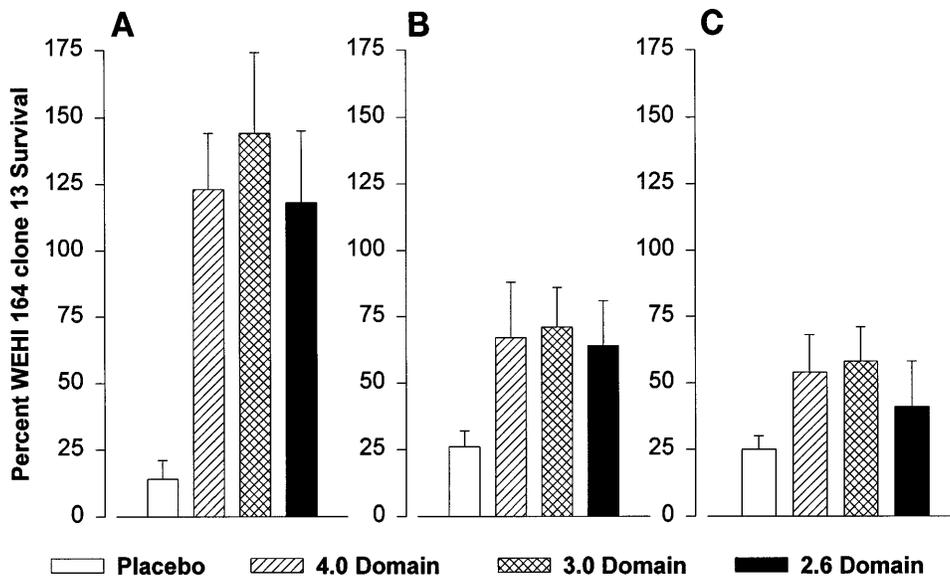


Fig. 6. Excess TNF- α neutralizing capacity in plasma from baboons treated with TNFR constructs. Serial dilutions [1:1 (A), 1:5,000 (B), and 1:10,000 (C)] of plasma obtained 90 min after *E. coli* infusions from baboons pretreated with the 3 constructs were incubated with WEHI 164 clone 13 cells, to which recombinant human TNF- α (310 pg/ml) was added. Plasma samples from baboons treated with the 3 constructs showed equivalent neutralizing capacity when coincubated with added TNF- α and TNF- α -sensitive cell line, WEHI 164 clone 13.

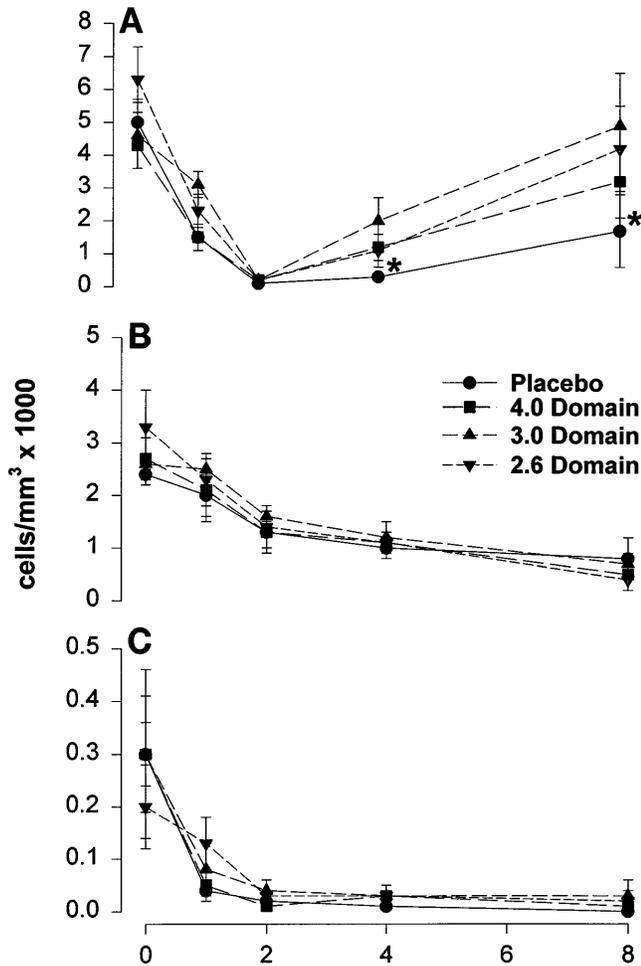


Fig. 7. Blood leukocyte changes after *E. coli* administration. Blood was obtained from baboons at intervals after bacterial administration, and total and differential white counts were determined. *E. coli* bacteremia produced a sustained monocytopenia and lymphopenia that were unaffected by TNF-bp treatment. Although all animals developed an early neutropenia, pretreatment with TNF-bp reduced duration of neutropenia, and neutrophil counts were significantly lower in placebo-treated animals than in TNF-bp groups at 4 and 8 h. A: neutrophils; B: lymphocytes; C: monocytes. * $P < 0.05$ vs. other groups.

Survival. Three of the four baboons pretreated only with placebo died or required euthanasia (for animal welfare reasons) within 5–30 h after *E. coli* challenge, and one animal survived all 21 days. In contrast, all but one of the 12 animals that received one of the TNF-bp constructs survived 21 days (11/12, 91%; $P < 0.05$ by Fisher's exact test). Groups treated with the 3.0-domain (4/4) and 4.0-domain (4/4) constructs had 100% survival, compared with baboons treated with 2.6 domain (3/4), which had 75% survival. At present, an explanation for the sole death in the one baboon receiving the 2.6-domain construct is not readily apparent, as the plasma TNF- α response in this animal (discussed below) was completely abolished and the proinflammatory cytokine response was not different from that in the surviving animals.

Plasma TNF- α and TNF neutralizing capacity. Administration of *E. coli* to the placebo-treated baboons resulted in the rapid appearance of TNF- α immunoactivity and WEHI 163 clone 13 bioactivity that peaked within 90 min and declined thereafter (Fig. 5). In contrast, in baboons treated with either the 4.0-, 3.0-, or 2.6-domain TNF-bp construct, no measurable TNF- α bioactivity was detected at any time point after the *E. coli* administration. Furthermore, in all of the 90-min plasma samples from baboons treated with *E. coli* and either the 4.0-, 3.0-, or 2.6-domain TNF-bp construct, excess TNF neutralizing capacity was present. In all cases, samples could be diluted 1:5,000 to 1:10,000 before one-half of the neutralizing capacity of 310 pg/ml added TNF- α was lost (Fig. 6).

Although treatment with the TNF-bp constructs eliminated all TNF bioactivity from the plasma, TNF- α immunoactivity remained (Fig. 5). The TNF- α ELISA employed can detect TNF- α bound to either the type I or II receptor but does so with reduced efficiency depending on the ratio of TNF- α to TNFR (28). Thus interpretation of the absolute levels of TNF- α immunoactivity in the circulation is difficult. However, the data suggest that treatment of the baboons with either the 4.0-, 3.0-, or 2.6-domain TNF-bp extends the plasma

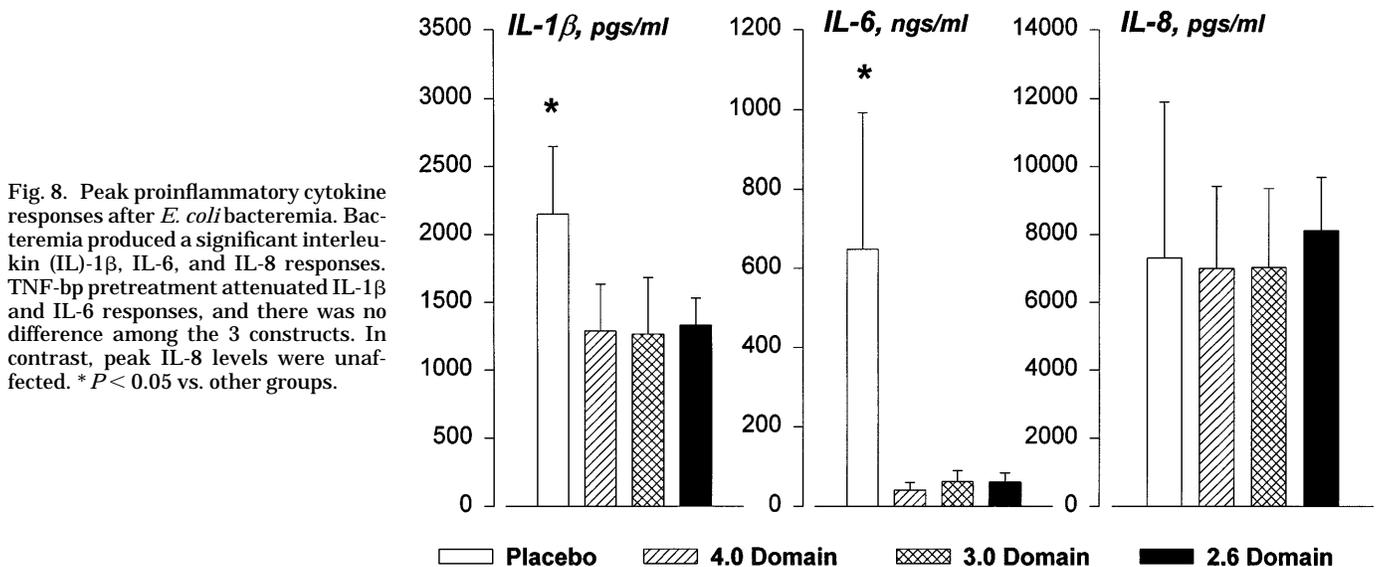


Fig. 8. Peak proinflammatory cytokine responses after *E. coli* bacteremia. Bacteremia produced a significant interleukin (IL)-1 β , IL-6, and IL-8 responses. TNF-bp pretreatment attenuated IL-1 β and IL-6 responses, and there was no difference among the 3 constructs. In contrast, peak IL-8 levels were unaffected. * $P < 0.05$ vs. other groups.

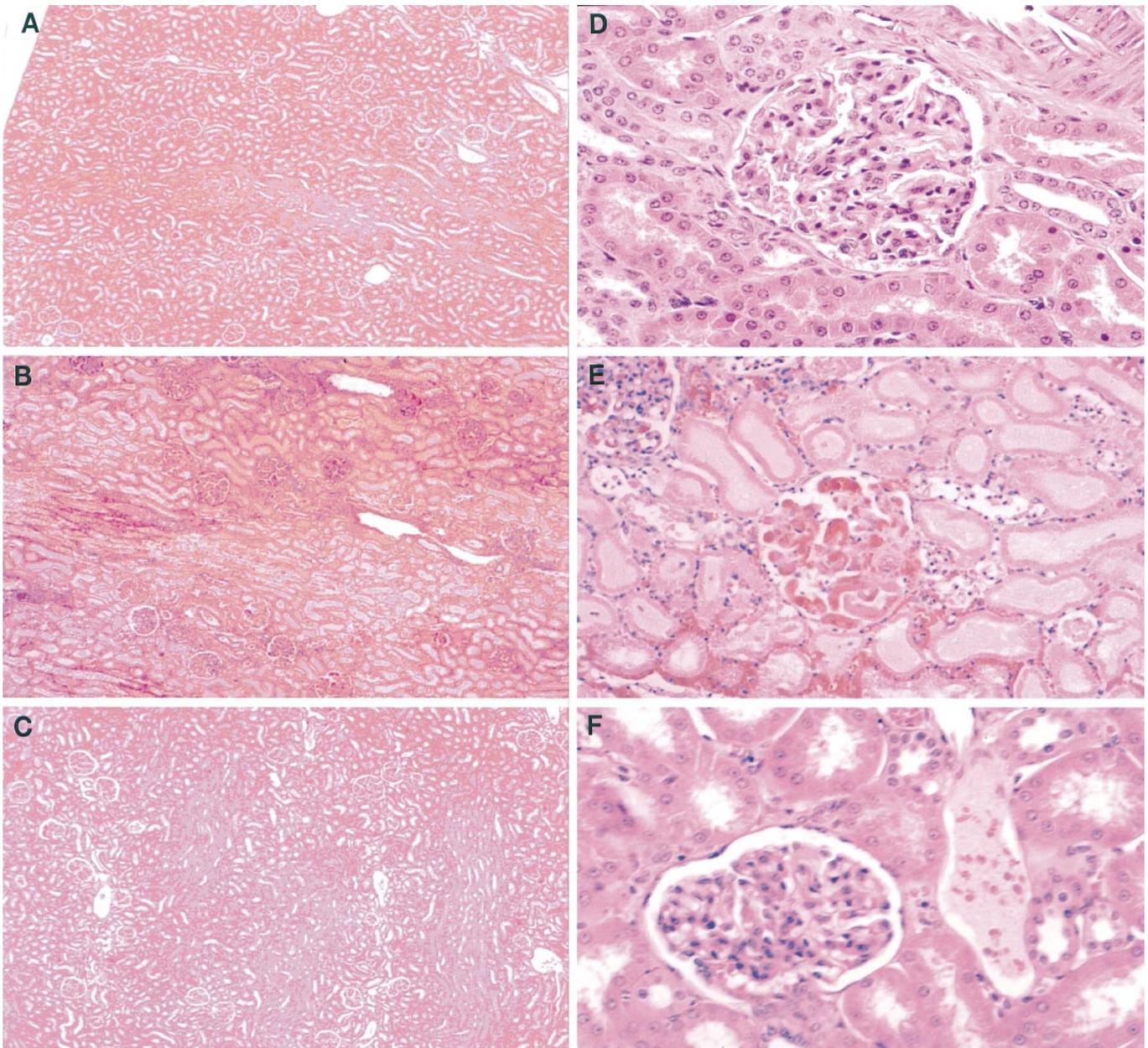


Fig. 9. Histopathological changes in kidneys from healthy and bacteremic baboons. Kidney specimens were obtained at necropsy, fixed in buffered Formalin, sectioned at 5 μm , and stained with hematoxylin and eosin. Original magnification $\times 100$ (A, B, C) and $\times 500$ (D, E, F). A and D: representative kidney specimen from a healthy baboon treated with 1 of the 3 TNF-bp constructs twice over a 42-day period. Appearance of tissue is essentially normal, with no evidence of vacuolization or subacute inflammation (baboon 95-4 treated with the 2.6-domain construct). B and E: kidney from a placebo-pretreated animal (baboon 95-33) that was euthanized after *E. coli* bacteremia because of animal welfare concerns. There is evidence of marked cortical and medullary necrosis and vasculitis. C and F: kidney from a 2.6-domain-pretreated animal (baboon 95-7) 21 days after *E. coli* bacteremia. There was only very modest evidence of subacute multifocal inflammation, and the kidney specimen appeared normal.

half-life of the molecule, although in a bound and biologically inactive form.

Other cytokines. Treatment with the TNF-bp significantly attenuated both the IL-1 β and IL-6 concentrations in the baboons, and there were no significant differences among the three constructs (Fig. 7). IL-8 concentrations were only modestly affected by the TNF-bp treatments.

Hematologic responses. *E. coli* administration in the baboon produces a profound leukopenia that is sus-

tained until the animal expires. Pretreatment with either the 4.0-, 3.0- or 2.6-domain TNF-bp construct did not prevent the neutropenia or lymphopenia that accompanied the response but significantly shortened the duration of neutropenia (Fig. 8). In fact, in baboons treated with either of the TNF-bp constructs, neutrophil counts were significantly higher at 4 and 8 h ($P < 0.01$) than in animals given placebo.

Histopathological changes. Kidneys from *E. coli* bacteremic baboons treated with placebo revealed moder-

ate-to-severe multifocal cortical necrosis with fibrin thrombi in the glomeruli (Fig. 9). In contrast, in the baboons treated with the 4.0-, 3.0-, and 2.6-domain TNF-bp, only mild, subacute purulent nephritis was generally noted. In an occasional animal, multifocal cortical necrosis was also observed (Table 4). In the one 2.6-domain TNF-bp-treated baboon that was euthanized for animal welfare concerns, severe diffuse renal cortical necrosis and widespread thrombosis and moderate vasculitis were observed (data not shown).

DISCUSSION

The present study confirms that pegylated TNFR constructs can be safely administered to healthy baboons at doses of 0.2–1 mg/kg body wt, have extended biological half-lives compared with monomeric TNFR I, and can block the pathological effects of a systemic TNF- α response secondary to *E. coli* bacteremia. There was no evidence of renal vacuolization secondary to the repeated administration of the constructs containing polyethylene glycol. When animals are pretreated, these TNFR constructs appear to be as effective as monoclonal antibodies against TNF- α , soluble TNFR, or TNFR immunoadhesins (14, 26, 28, 29) at improving survival, attenuating a proinflammatory cytokine response, and preventing a sustained neutropenia (29). However, the quantities required to neutralize a systemic TNF- α response in this model appear to be significantly less than for either monomeric TNFR I (28) or for monoclonal antibodies against human TNF- α (14, 26). This is confirmed by the *in vitro* studies (Fig. 2) in which the dimeric TNF-bp construct was ~20 times more effective at neutralizing TNF- α as monomeric forms of the construct. Although a dose-response curve was not generated in the present study, the quantities of dimeric TNF-bp required to confer survival appeared to be similar to these recently reported by us for TNF-receptor immunoadhesins (29). Furthermore, the ability to reduce other proinflammatory cytokines, such as IL-1 β and IL-6, as well as to promote the recovery of blood neutrophils was comparable to that seen with TNF-receptor immunoadhesins (29).

It should be noted that the *E. coli* bacteremia model in *Papio* was designed to test the efficacy of these preparations in a model of TNF- α -mediated injury. Considerable controversy exists regarding the suitability of primate and rodent models of bacteremia or endotoxemia to represent human sepsis or systemic

inflammatory response syndromes (7). In fact, the markedly improved outcomes observed with TNF- α and IL-1 inhibitors in identical primate models of bacteremic or endotoxemic shock have not been reproduced in human sepsis. The majority of results from clinical studies with TNF- α or IL-1 inhibitors in sepsis syndromes have either been equivocal (1, 2, 9, 10) or have, in one case, shown increased mortality (8).

More recently, the primary recommended use for these TNF- α inhibitors has shifted away from acute inflammatory diseases such as sepsis or reperfusion injury to more chronic inflammatory processes dependent on TNF- α . The most promising clinical data have come from investigations in rheumatoid arthritis, where preliminary results suggest that significant abrogation of disease progression can be achieved with protein-based inhibitors of TNF- α activity (4, 16, 18). However, clinical studies are also underway for inflammatory bowel disease, acquired immunodeficiency syndrome cachexia, graft vs. host disease, and multiple sclerosis.

The *E. coli* bacteremic primate model offers several unique advantages to evaluate both efficacy and pharmacokinetics of such TNF-receptor constructs, independent of its relevance to human sepsis. The *E. coli*-shock model has been studied for almost 10 yr by several independent research groups. The accumulated database is large, and data suggest that mortality and the pathological responses are dependent almost exclusively on exaggerated TNF- α or IL-1 production. Administration of either monoclonal antibodies, soluble TNF receptors, TNF-receptor constructs, or TNF-receptor immunoadhesins that inhibit TNF- α bioactivity prevent the lethal consequences (5, 11, 12, 24, 26–28).

Both the size of the animal and the cross-reactivity of *Papio* cytokines with human reagents make the species ideal for invasive monitoring and the multiple blood sampling that is required for the successful conduct of the studies. Furthermore, *Papio* are indigenous to many regions of the world, their numbers are not endangered, and, unlike many other primates, the species does not readily harbor several human pathogenic viruses, including herpes simplex B, hepatitis B or C, human immunodeficiency virus, or simian immunodeficiency virus. Like in all nonhuman primates, immunogenic responses to administered human proteins may develop in the baboon, and extrapolation of immunogenicity in the primate to that in human must be done with caution. However, we have recently cloned the extracellular domain of the baboon TNFR I and have noted that the amino acid sequence differs from the extracellular domain of human TNFR I at only five residues (unpublished observations).

The desire to abrogate TNF- α bioactivity over extended periods, often exceeding several months, has increased the need to develop less-expensive protein-based therapies with extended biological half-lives and reduced immunogenicity. Pegylated TNFR constructs (like those described here) meet the former criteria of being less expensive to manufacture than monoclonal antibodies or immunoadhesins and having extended biological half-lives. Although the capacity to neutral-

Table 4. *Histological changes in the kidney during E. coli bacteremia*

Median (Range)	Cortical Necrosis	Medullary Necrosis	Thrombosis	Subacute Inflammation
Placebo	3 (0–4)	0 (0–4)	4 (1–4)	0 (0–1)
4.0 Domain	0 (0)	0 (0)	0 (0)	2 (1–2)
3.0 Domain	0 (0)	0 (0)	0 (0)	1 (1–2)
2.6 Domain	0 (0–4*)	0 (0)	0 (0–4*)	2 (0–2)

Scoring system was 0–4, where 0 represented absence of changes, 4 represented widespread severe histological changes; *n* = 4 baboons/group. * Value from the 1 animal that required euthanasia because of impending death.

ize TNF- α and improve outcome in a model of TNF- α -mediated injury did not appear to be significantly altered by structural modifications of the TNFR construct, immunogenicity and subsequent plasma half-life were affected. It should be noted that the antibodies that developed were neither neutralizing nor cytotoxic in *in vitro* assays. However, under *in vivo* conditions, there was a good correlation between the presence of an antibody response and the shortening of the terminal half-life. This suggests that antibody-TNF-bp interactions led to the more rapid clearance of the TNF-bp constructs at plasma levels <500 ng/ml.

Modification of the number of structural domains of the construct tended to decrease, but not eliminate, the immunogenicity of the preparations. The onset and magnitude of the immunogenic response appeared to be dependent on the number of functional domains as the polyethylene glycol component of the constructs remained constant.

The clinical significance of the antibody response in humans and primates is unclear. Antibody responses have been reported in humans receiving anti-TNF- α monoclonal antibodies (2) and TNF-receptor immunoadhesins (8). In the present study, animals that developed an antibody response after the first dose had a significantly shorter terminal half-life after their second administration. Thus such findings suggest that antibody responses may reduce the biological half-life and, thus, therapeutic efficacy of the constructs, and dose adjustments may be required. However, there did not appear to be any adverse clinical response to the presence of the antibodies when the constructs were administered a second time. Therapeutic efforts to modify such constructs to reduce immunogenicity, without significantly affecting half-life or efficacy, are aimed primarily at reducing the need for increasing dose adjustments, rather than the risk of adverse reactions.

Nevertheless, the studies suggest that such dimeric, pegylated TNF-receptor constructs can be generated that are effective inhibitors of TNF- α bioactivity. The challenge in the future is to design such constructs with reduced immunogenicity and increased biological half-lives.

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