Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 8, pp. 793–802, 1987
Printed in Great Britain

0731–7085/87 $3.00 + 0.00
Pergamon Journals Ltd.

ELISA for quantitation of tumor necrosis factor-α in serum*

W. S. PRINCE,[1] K. J. HARDER,[2] S. SAKS,[2] B. R. REED,[1] A. B. CHEN[1]† and A. J. S. JONES[1]

[1] *Departments of Medicinal and Analytical Chemistry and* [2] *Clinical Research Genentech, Inc., 460 Pt. San Bruno Blvd, South San Francisco, CA 94080, USA*

Abstract: A sensitive and precise ELISA has been developed for the quantitation of recombinant Tumor Necrosis Factor-Alpha (rTNF-α) in undiluted sera. Affinity purified rabbit antibody was used as capture antibody and mouse monoclonal antibody labelled with horseradish peroxidase was used as the second antibody in a sandwich ELISA. The assay range was from 50 to 2000 pg/ml and the relative standard deviation was 8% or less for both interassay and intra-assay precision studies. Recovery of rTNF-α added to 10 different human and 10 different monkey sera ranged from 81 to 102% and 100 to 120% of the expected value, respectively.

This ELISA has been used to measure serum rTNF-α levels in over 60 patients in Phase I Clinical Trials treated with rTNF-α. The levels in a representative, pharmacokinetic study showed low variability between 8 patients receiving intravenous bolus administration of 100 μg rTNF-α/m^2. The ELISA results correlated well with TNF bioassay data with a mean specific activity of 2.5×10^7 U/mg.

Keywords: *Enzyme immunoassay; tumor necrosis factor-alpha; serum effects.*

Introduction

The need to detect and quantitate trace levels of recombinant DNA derived proteins in serum or plasma in preclinical studies is well known [1]. Since the toxicities of these recombinant proteins are unknown, Phase I Clinical Trials (safety trials) are initiated at low concentrations of the pharmaceutical followed by increasing doses. An assay must therefore be sensitive, specific and applicable to clinical specimens.

Carswell *et al.* [2] discovered a number of factors which caused haemorrhagic necrosis of various tumors in mice with no effect on the host. One of these factors, tumor necrosis factor-alpha, has been cloned and expressed in *E. coli* [3, 4], and has also been purified from HL-60 cells induced by a phorbol ester [5]. Recently rTNF-α was shown to have antiviral effects *in vitro* [6, 7] and to synergize with interferons [7]. Because of its activity of causing haemorrhagic necrosis and in some cases complete regression of transplanted

*Presented at the "International Symposium on Immunoassays", November 1986, Arlanda, Sweden.
†To whom correspondence should be addressed.

tumors in mice [2, 8] it has been under evaluation as a possible treatment for patients with malignant diseases and Phase I trials have begun [9–11]. This report describes our studies and the experiments which were necessary to show that the ELISA assay was suitable for quantitation of rTNF-α in serum.

Materials and Methods

Affinity purified rabbit anti-rTNF-α

Rabbits were immunized with purified rTNF-α (produced at Genentech, Inc.) according to established procedures [12]. The rabbits were bled, sera pooled and the immunoglobulin fraction was obtained by ammonium sulphate fractionation. The immunoglobulin fraction was further purified by affinity chromatography on a column of rTNF-α coupled to cyanogen bromide (CNBr) activated Sepharose 4B (Pharmacia, Piscataway, NJ). The antibodies specific for rTNF-α were eluted with 0.2 M glycine-HCl, 0.5 M NaCl, pH 2.8. A portion of this antibody was then coupled to horseradish peroxidase (HRP) by the method of Nakane [13]. The column of rTNF-α was re-used on several occasions which suggests that immunological reactivity of rTNF-α was retained after several acid elution cycles.

Rabbit anti-rTNF-α affinity column

Ten milligrams of affinity purified rabbit anti-rTNF-α were coupled to 10 ml of Affigel 10 (BioRad Laboratories) according to manufacturer's recommendations. Briefly, Affigel 10 was successively washed with isopropanol and distilled water and then added to 5 ml of anti-rTNF-α. The gel and antibody were mixed "end over end" at 4°C for 12 h. Excess liquid was removed and unconjugated sites were blocked with 1.5 ml of 1 M glycine ethyl ester. The gel was then washed with PBS and poured into a 1 × 30 cm column. From absorbance measurements at 280 nm, it was estimated that 98% of the antibodies were coupled to the gel.

Monoclonal antibodies to TNF-α

These were prepared and characterized as described by Bringman *et al.* [14]. Two monoclonals, B and D, which neutralized the cytolytic activity of rTNF-α were used. Monoclonal D was labelled with HRP by the method of Nakane [13].

Animal and human sera

Murine sera were obtained from Pel Freez (Rogers, Arkansas) and from BDF-1 mice. Sera were also collected from cynomolgus, rhesus and squirrel monkeys. Human sera were obtained from Peninsula Blood Bank, Burlingame, California.

Bioassay activity of rTNF-α

This was measured as cytolytic activity on murine connective tissue L-M cells as described by Kramer and Carver [15]. Briefly, two-fold dilutions of rTNF-α standard and samples were applied into L-M cells seeded in wells of flat bottom microtitre plates. After incubation at 39°C for 18–20 h, the medium was removed and the plates were stained with 0.5% crystal violet. The quantitation of cytolytic activity was based on crystal violet dye uptake of residual viable cells and compared to rTNF-α standard (Std). The endpoint (EP) corresponding to the 50% cytotoxicity absorbance value was used to calculate sample activity by the formula:

$$\text{Sample Activity (U/ml)} = \text{Standard Activity (U/ml)} \times \frac{(\text{Sample EP} - \text{Std EP})}{2}$$

ELISA protocol

In each well of a 96-well mictotitre plate (NUNC Immunoplate I, NUNC Kamstrup, Denmark) was applied 100 μl of a 0.5 μg per ml solution of affinity purified rabbit anti-rTNF-α diluted in 0.05 M sodium carbonate pH 9.6. The plate was sealed with plate sealing tape and incubated at 2–8°C overnight. Excess coating antibody was then removed, plates washed (Dynawasher II, Dynatech) three times with 200 μl of 0.01 M phosphate buffered saline pH 7.4 (PBS) containing 0.05% Tween 20 (Tween), and subsequently blocked with 200 μl of 0.5% bovine serum albumin (BSA) in PBS:Tween for 1–2 h at ambient temperature with agitation (Bellco orbital shaker, Bellco, Vineland, NJ), and then washed. Serum samples (100 μl), rTNF-α standards (50–2000 pg/ml) and controls diluted in foetal bovine serum with 0.01% thimerosal, were added to appropriate wells of the plate. The sealed plate was incubated for 12–18 h at 2–8°C. After washing, 100 μl of horseradish peroxidase (HRP) conjugated mouse monoclonal D anti-rTNF-α, in PBS:BSA, was added to each well. The plate was sealed and incubated for 2 h at ambient temperature with agitation, and then washed. To each well, 100 μl of substrate solution (4.0 μg per ml orthophenylenediamine in 0.2 M sodium phosphate, 0.1 M sodium citrate, pH 5.0) was added, and the plate was incubated in the dark at ambient temperature for 30 min. The reaction was stopped by adding 100 μl of 4.5 N sulphuric acid to each well, and absorbances of each well were read at 492 nm (Multiscan II, Flow). The data were reduced using a 4-parameter logistic curve fitting program [16].

For studies using the same species antibody for coat and second antibody, HRP labelled rabbit anti-rTNF-α was substituted for HRP-labelled mouse monoclonal D. In the mouse–mouse system microtitre plates were coated with 0.5 μg/ml mouse monoclonal B instead of rabbit anti-rTNF-α.

Analyses on polyacrylamide gel (12.5%) were performed as described by Laemmli [17] and immunoblots were performed as described by Towbin *et al.* [18].

Results

Affinity purified polyclonal rTNF-α assay

An initial assay for rTNF-α in aqueous buffers, using foetal bovine serum (FBS) as diluent for the standard, was developed using affinity purified rabbit antibodies. At rTNF-α concentrations of 3.12 ng/ml and less, PBS:BSA buffer resulted in lower ELISA reactivity than that observed in FBS. Other protein-containing buffers were tried but FBS diluent for rTNF-α yielded the best results (data not shown). To determine if rTNF-α could be assayed in undiluted sera, standard curves of rTNF-α in various animal sera were prepared and compared to the FBS standard curve. The curves were not superimposable (Fig. 1). Therefore, the quantification of rTNF-α in these sera could be incorrect (assuming FBS values are correct). Higher values would suggest the presence of endogenous TNF, whereas lower values would suggest interfering substances. Generally when human and other sera were tested undiluted, background TNF-α levels were high in this system (see Table 1).

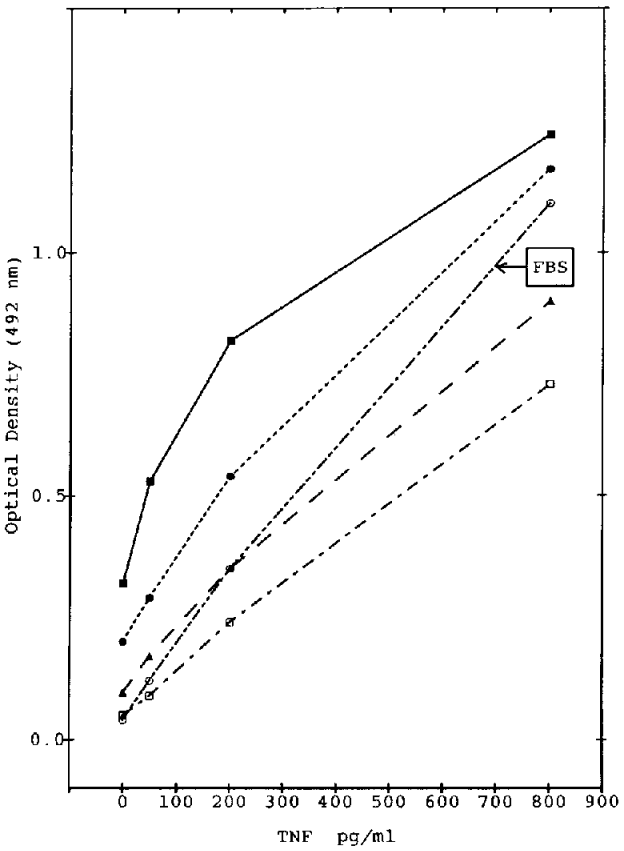

Figure 1
Standard curves of rTNF-α added to animal sera using rabbit antibody as coat and conjugate. ■——■ mouse number 1426; ●- - -● mouse BDF1; ○— - - - - -○ foetal bovine; ▲- - -▲ cynomolgus; and rhesus □— - - - - -□.

Table 1
rTNF-α ELISA background levels[a] for normal human sera

Serum number	Coat antibody Conjugated antibody	PAb[b] PAb	MAb[c] MAb	PAb MAb
1		342	<25	<25
2		169	224	<25
3		297	<25	<25
4		<25	<25	<25
5		1075	<25	<25
6		<25	<25	<25
7		329	<25	<25
8		<25	<25	<25
9		151	<25	<25
10		136	62	<25

[a] Each value in pg/ml is the mean of duplicates.
[b] PAb:Rabbit polyclonal antibody.
[c] MAb:Mouse monoclonal antibody.
Each ELISA system was optimized for optimum reactivity. The ten human sera were then assayed undiluted in each separate ELISA. Standard curve for each system was rTNF-α in FBS.

High molecular weight (HMW) component

One bovine calf serum in particular seemed to have high TNF-like reactivity and was examined to determine the origin of this "TNF" ELISA reactivity. This serum was applied to an affinity column made with affinity-purified rabbit anti-rTNF-α coupled to Affigel 10. The initial, flow-through and eluted fractions were analyzed by polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis. The Coomassie Brilliant Blue stained gel indicated that the initial and flow-through lanes were quite similar and appear to be a normal serum profile. The eluted fraction contained a major band of high molecular weight (>92 kilodaltons, Fig. 2). No TNF-like protein band was observed co-migrating with the rTNF-α control (molecular weight 17,000; see ref. 3).

The immunoblot of a companion gel transferred to nitrocellulose also suggested that there was no TNF detectable in any of the fractions of the bovine serum. (It is not likely that the acid elution conditions alter immunoreactivity of rTNF-α since rTNF-α coupled to affinity columns has been repeatedly subjected to acid conditions during purification of antibodies (see Methods). Acid elution conditions have also been used to

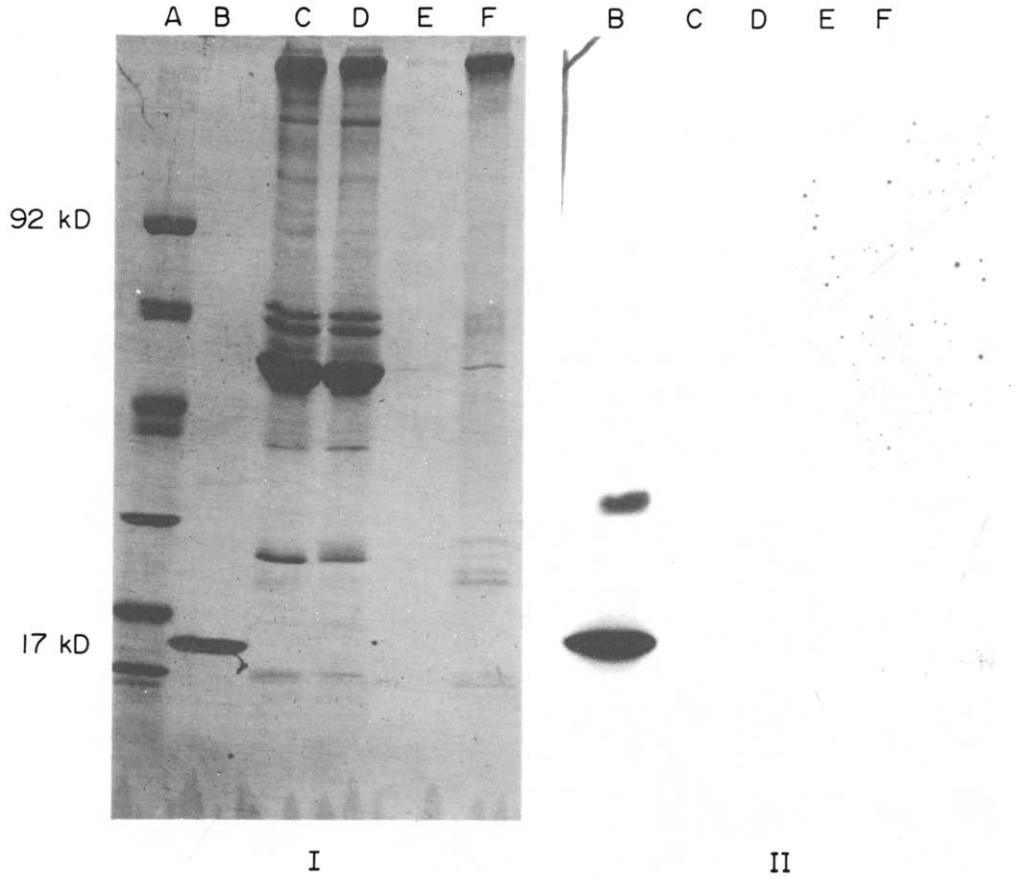

Figure 2
Binding of high molecular weight (>92 kDa) serum protein to rabbit anti-rTNF-α affinity column. I. Coomassie stained gel II. Immunoblot using rabbit anti-rTNF-α and ^{125}I-labelled Protein A. A. Molecular weight standards (BioRad); B. rTNF-α ; C. Bovine serum before application; D. Flow through; E. 1 M NaCl wash; and F. Elution with 0.2 M glycine-HCl pH 2.4.

purify rTNF-α [14].) However, there was some reactivity in the high molecular weight (HMW) fraction. This experiment was repeated with a goat serum which also demonstrated high TNF-like activity in undiluted serum. The similar finding of no band co-migrating with rTNF-α by PAGE and reactivity of rabbit anti-rTNF-α with a HMW fraction was observed (unpublished data). From molecular weight markers the HMW fraction was estimated to be 160,000 daltons.

Mouse monoclonal rTNF-α assay

Since various sera seemed to have different background levels in the rTNF-α ELISA assay using rabbit polyclonal antibodies as coat and second antibody, a monoclonal antibody (MAb) configuration was evaluated. Two MAbs which neutralized cytolytic activity of rTNF-α were used [14]. Monoclonal antibody B was used as capture antibody and MAb D HRP-labelled as second antibody. This assay was able to detect rTNF-α diluted in FBS to the same level of sensitivity as the rabbit polyclonal based assay.

rTNF-α was then added to ten human and ten monkey sera and two-fold dilutions were made with each serum. The dilution curves as quantitated from a standard curve of rTNF-α in FBS are shown for the sera in Figs 3A and 3B respectively. There was quite a wide variability of response. In 8 human sera and 7 monkey sera the values obtained were below that expected and one human and one monkey sera were more than the expected values. The ranges of recovery of rTNF-α values were 34 to 196% for human sera and 62 to 374% for monkey sera. Thus this combination of mouse monoclonals was not adequate for quantitating rTNF-α accurately in undiluted sera at the concentrations tested.

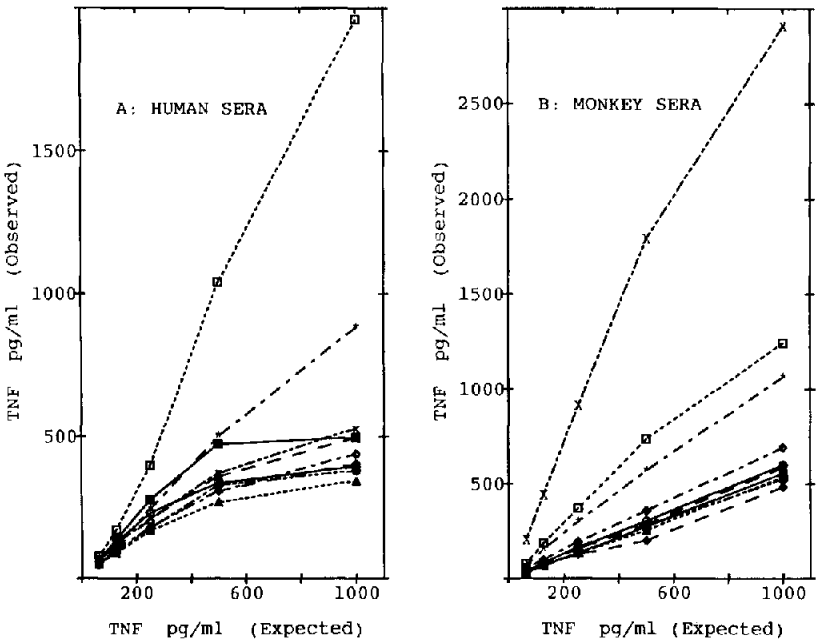

Figure 3
Dilution curves using mouse monoclonal antibody B as coat and mouse monoclonal antibody D, HRP-labelled as conjugate. A: Human sera; B: Monkey sera. rTNF-α was added to each serum to a nominal concentration of 1000 pg/ml. Two-fold dilutions were then made in each original serum and assayed.

Polyclonal:monoclonal rTNF-α assay

When a mixture of the two systems was used, i.e. polyclonal rabbit antibody as coat and mouse monoclonal D HRP-labelled as second antibody, background levels were negligible. Table 1 shows the data obtained when ten normal human sera were tested using the indicated combinations of rabbit polyclonal and mouse monoclonal antibodies. Figures 4A and 4B show the recovery and linearity of dilution of the same ten human and ten monkey sera shown in Figs 3A and 3B. The curves for all the sera nearly overlap.

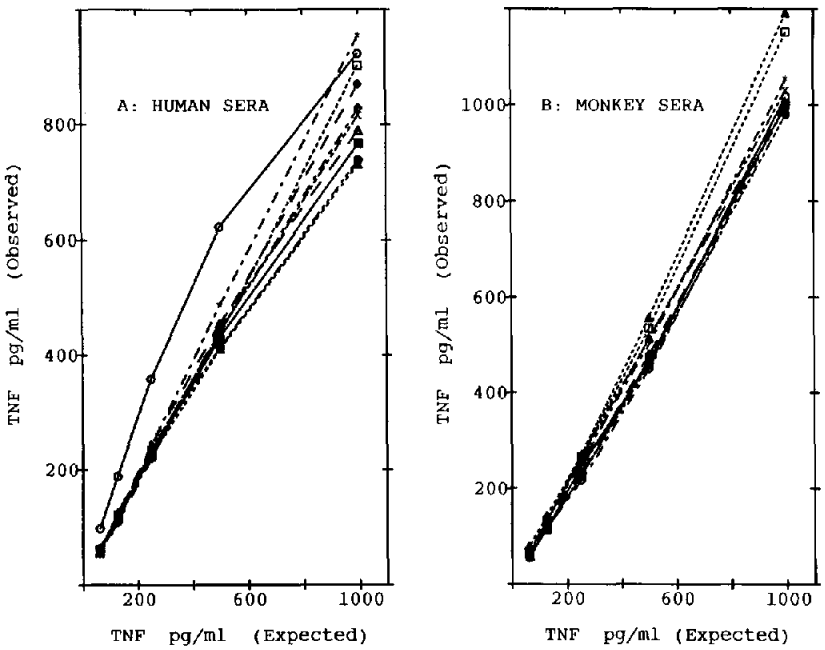

Figure 4
Dilution curves using rabbit anti-rTNF-α as coat and mouse monoclonal antibody D, HRP-labelled as conjugate. A: human sera; B: monkey sera. rTNF-α was added to each serum to a nominal concentration of 1000 pg/ml. Two-fold dilutions were then made in each original serum and then assayed. The ten monkey and ten human sera were the same as for Fig. 3.

Furthermore, the ranges of rTNF-α recoveries were very good being 81 to 102% for human sera and 100 to 120% for monkey sera. The linearity of dilutions in the individual sera were good with correlation coefficients of observed to expected values of approximately 0.999. rTNF-α was also added to human blood at levels expected during clinical trials, serum obtained and diluted with FBS into assay range. Recovered values were similar to expected (Table 2). Therefore this assay configuration can be used to accurately measure rTNF-α in serum, both undiluted and diluted in FBS.

The precision of the assay when quantitating controls (rTNF-α added to FBS) was good, intra-assay and interassay relative standard deviation (RSD) being 8% or less (Tables 3A and 3B). Minimum detectable concentration according to Rodbard [16] is equivalent to the concentration corresponding to the mean absorbance of the zero standard plus twice the standard deviation and was determined to be 12 pg/ml (Table 3C). Cross reactivity of rTNF-α and recombinant interferon-gamma was not detectable (<0.0002%).

Table 2
Determination of rTNF-α in serum and plasma

Donor	Serum[a] 0 h	Serum[a] 24 h	EDTA plasma 0 h	EDTA plasma 24 h	Citrate plasma 0 h	Citrate plasma 24 h
1	ND	7.7[b]	6.9	7.3	7.8	4.3
2	5.4	10.1	9.9	8.5	8.5	8.3
3	8.7	7.2	5.8	5.5	6.2	6.4
4	8.0	6.7	4.6	4.0	4.8	4.6
5	8.3	7.2	4.9	4.6	5.5	5.2
6	7.1	7.4	4.0	4.3	4.4	4.4
7	7.2	6.8	6.3	6.8	3.0	3.9
8	6.8	6.8	5.3	2.3	1.3	1.4
9	4.0	5.5	6.2	5.8	5.0	4.6
10	7.0	6.2	5.6	4.9	ND	ND
Mean	6.94	7.16	5.95	5.40	5.17	4.79
SD	1.47	1.21	1.63	1.8	2.23	1.87

[a]rTNF-α (10 ng/ml) was added to freshly collected whole blood. The blood was then processed to serum or plasma either immediately (for serum, blood was allowed to clot) or after 24 h incubation at 4°C. Samples were then diluted with FBS into assay range.
ND = Not done; [b]Values are in ng/ml and are corrected for the dilution necessary to attain assay range.

Table 3

A. *INTRA-ASSAY Precision*[a]

Control	rTNF-α pg/ml	Percent RSD	N
Low	51.7	7.7	19
Mid	261	4.2	19
Hi	1375	3.1	18

B. *INTERASSAY Precision*[b]

Control	rTNF-α pg/ml	Percent RSD	
Low	48	8.1	10
Mid	218	7.1	11
Hi	1144	7.4	11

C. *MINIMUM DETECTABLE DOSE*[c]

Mean absorbance of FBS from 20 replicates	0.0164
SD	0.0047
Mean + 2 standard deviations	0.0258
Minimum detectable dose (rTNF concentration at 0.0258)	12 pg/ml

[a]Control samples (rTNF-α added to FBS) were assayed within one mictotitre plate on more than one occasion with similar results.
[b]Control samples were assayed on 10 or more different microtitre plates.
[c]Minimum detectable dose calculated according to Rodbard *et al.* [16].

Clinical samples

Plasma or serum. rTNF-α (10 ng/ml) was added to 10 freshly collected blood specimens which were processed either as plasma (EDTA or citrate) or serum. The red blood cells, for plasma, or the fibrin clot for serum, was removed either immediately or after a 24-h incubation at 4°C. Table 2 shows the highest mean recovery of added rTNF-α was obtained with serum and processing delays did not hinder recovery ($P > 0.1$).

Significant differences were observed when comparing plasma to serum values ($P = 0.01$). Thus rTNF-α values in treated patients were determined on serum samples.

This ELISA has been used to measure rTNF-α levels in over 60 patients in Phase I Clinical Trials treated with rTNF-α administered by IV, SC and IM routes. Serum samples which contained rTNF-α values higher than the range of the standard curve were diluted in FBS, re-assayed and levels corrected for dilution. The mean rTNF-α levels determined by ELISA and bioassay for 8 patients receiving intravenous bolus administration of 100 μg rTNF-α/m^2 are plotted in Fig. 5. The ELISA data correlated well with bioassay data. The average RSD for the bioassay activity, at the measured timepoints, was 55% (range 33 to 68%). For ELISA activity the average RSD was 58% (range 37 to 82%). The specific activities of rTNF-α at the measured timepoints ranged from 1.9 to 4.0 × 10^7 U/mg with a mean of 2.5 × 10^7 U/mg similar to values reported for rTNF-α (3–5).

Discussion

An assay for quantitation of rTNF-α in undiluted serum has been developed, validated and used in clinical trials. The assay used rabbit antibody as capture antibody

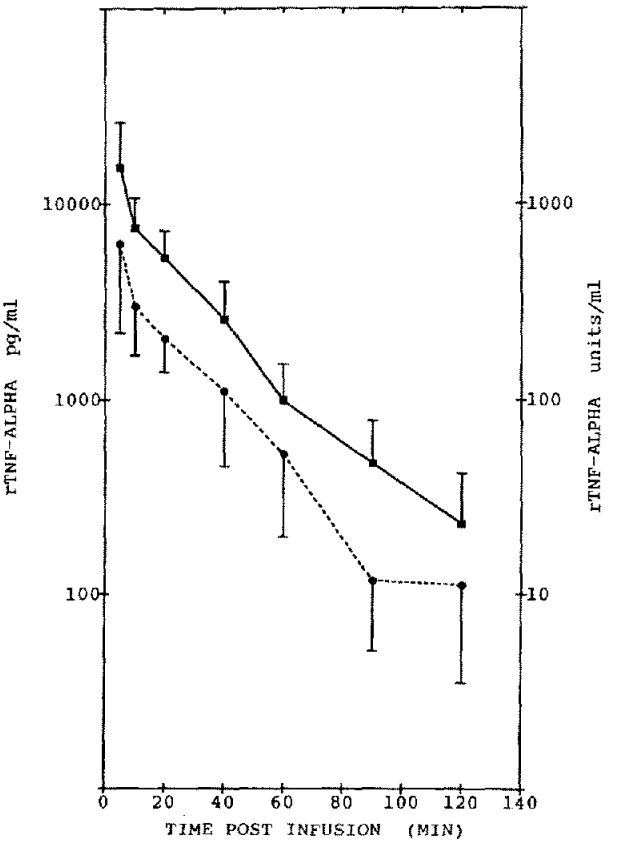

Figure 5
Pharmacokinetic profile of ELISA and cytolytic values of 8 patients treated with 100 μg rTNF-α/m^2. ■ —, ELISA values (pg/ml); ● —, bioassay values (Units/ml).

and mouse monoclonal antibody as second antibody. Other assay configurations evaluated were not capable of accurately quantitating rTNF-α. For example, high serum background levels were observed in the double rabbit polyclonal system and may have been related to the high molecular weight protein observed. During development of an assay for another protein using rabbit polyclonal antibody as capture antibody, high serum background levels were eliminated by the addition of rabbit IgG to the sera prior to assay [19]. Measurement of rTNF-α values in the double monoclonal system were mainly lower than expected. Under-recovery may result from the presence of binding protein(s) in serum which inhibit binding of rTNF-α to the second monoclonal antibody. This explanation was supported by the fact that this inhibition was not seen when the monoclonal system was used to quantitate rTNF-α in aqueous buffers. The fact that several assays for the quantitation of proteins in serum also use a polyclonal:monoclonal system [20, 21] suggest that these investigators may have encountered similar problems.

Acknowledgements: The authors wish to thank Bob Wert and the Clinical Assay Services Group of Genentech for ELISA and Bioassays and the Genentech Word Processing Group for the typing of this manuscript.

References

[1] R. Kurzrock, M. G. Rosenblum, J. R. Quesada, S. A. Sherwin, L. M. Itri and J. U. Gutterman, *J. Clin. Oncol.* **4**, 1677–1683 (1986).
[2] E. A. Carswell, L. J. Old, R. I. Kassel, S. Green, N. Fiore and B. Williamson, *Proc. Natl. Acad. Sci. USA* **73**, 3666–3670 (1975).
[3] D. Pennica, G. E. Nedwin, J. S. Hayflick, P. H. Seeburg, R. Derynck, M. A. Palladino, W. J. Kohr, B. B. Aggarwal and D. V. Goeddel, *Nature, Lond.* **312**, 724–729 (1984).
[4] T. Shirai, H. Yamaguchi, H. Ito, C. W. Todd and R. B. Wallace, *Nature, Lond.* **313**, 803–806 (1985).
[5] B. B. Aggarwal, W. J. Kohr, P. E. Hass, B. Moffat, S. A. Spencer, W. J. Henzel, T. S. Bringman, G. E. Nedwin, D. V. Goeddel and R. N. Harkins, *J. Biol. Chem.* **260**, 2345–2354 (1985).
[6] J. Mestan, W. Digel, S. Mittnacht, H. Hillen, D. Blohm, A. Möller, H. Jacobsen and H. Kirchner, *Nature, Lond.* **323**, 816–819 (1986).
[7] G. H. W. Wong and D. V. Goeddel, *Nature, Lond.* **323**, 819–822 (1986).
[8] M. R. Ruff and G. E. Gifford, in *Lymphokines* Vol. 2, (Pick, E., Ed.) 235–275, Academic Press, NY (1981).
[9] M. B. Blick, S. A. Sherwin, M. G. Rosenblum and J. U. Gutterman, Abstract in *Proc. Amer. Soc. Clin. Oncol.* **5**, 14 (1986).
[10] S. E. Salmon and L. Young, Abstract in *Proc. Amer. Soc. Clin. Oncol.* **5**, 222 (1986).
[11] M. Pfreundschuh, T. Steinmetz, R. Gähl, M. Schaadt and V. Diehl, Abstract in *6th Int'l Congress Immunol.* Toronto, Canada, p. 546 (1986).
[12] D. H. Campbell, J. S. Garvey, N. E. Cremer and D. H. Sussdorf, in: *Methods in Immunology: A Laboratory Text for Instruction and Research* W. A. Benjamin, New York (1970).
[13] Nakane, P. K., in: *Immunoassays in the Clinical Laboratory* pp. 81–87. Alan R. Liss, New York (1979).
[14] T. S. Bringman, C. V. Benton and B. B. Aggarwal. 13th International Congress of Biochemistry, 25–30 August. Amsterdam, The Netherlands (1985).
[15] S. M. Kramer and M. E. Carver, *J. Immunol. Methods* **93**, 201–206 (1986).
[16] D. Rodbard, P. J. Munson and A. De Lean, in: *Radioimmunoassay and Related Procedures in Medicine*, Int'l Atomic Energy Agency, Vienna 1, 469–504 (1978).
[17] U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
[18] H. Towbin, T. Staehelin and J. Gordon, *Proc. Nat'l. Acad. Sci. USA* **76**, 4350 (1979).
[19] L. N. Bald, M. D. Geier, D. Drolet, G. Bennett, A. B. Chen, C. Lucas and P. Johnston, Manuscript in preparation.
[20] C. L. Bennett, N. J. Vogelzang, M. J. Ratain and S. D. Reich, *Cancer Treatment Reports* **70**, 1081–1084 (1986).
[21] R. John, R. Henley and D. Shankland, *Clin. Chem.* **32**, 2066–2069 (1986).

[*Received for review 21 January, 1987*]