Development of a sensitive ELISA for the quantification of human tumour necrosis factor-α using 4 polyclonal antibodies

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ABSTRACT. Despite the availability of many assays to measure concentrations of tumour necrosis factor alpha (TNF-α) in body fluids, these assays often lack specificity or sensitivity and are often of questionable reliability, resulting in inconsistent results. Therefore, we have developed an ELISA that is sensitive, reliable and not susceptible to disturbances by interfering substances such as heterophilic antibodies. The assay involves a combination of four polyclonal antibodies. The antibodies, which capture the analyte, were raised in chicken and the trapping anti-analyte antibodies were raised in rabbit. The immobilization of capture antibodies was achieved *via* a coating antibody raised in a duck against chicken IgY and the recognition of trapping antibodies was achieved by a detection antibody raised in a goat against rabbit IgG and labelled with HRP. The analytical and functional sensitivities of the ELISA are 8 pg/mL and 13 pg/mL, respectively. The assay showed good precision and, in contrast to our in-house RIA, excellent parallelism in serial dilutions. The recovery of TNF-α spiked to plasma samples ranged from 97% to 119%. Comparison of the newly developed, sensitive ELISA with our in-house RIA showed that the median TNF-α value obtained by RIA (range: 0.095-10.0, median 0.578 ng/mL) was found to be 1.5-2 times higher than that obtained with the ELISA (range 0.008-5.84, median 0.213 ng/mL). Spearman correlation was 0.755 ($p < 0.0001$). In addition, analysis of the TNF-α concentrations in blood from healthy individuals and from patients suffering from tuberculosis, with RIA and ELISA, showed the same differences although TNF-α levels obtained with ELISA were lower. We feel that this ELISA is a major improvement compared to the currently available assays for TNF.

Keywords: TNF-α, ELISA

The cytokine tumour necrosis factor-alpha (TNF-α) plays an important role in host defence in health and disease [1-4]. It is a 17 kDa polypeptide produced by macrophages and other cells [2]. Bioactive TNF-α is a homotrimeric protein [5, 6]. Two membrane receptors, proteins of 55 kDa and 75 kDa mediate and regulate most TNF activities [7-9]. After cleavage, they are released as soluble forms (sTNF-R1 and sTNF-R2) that contribute to the regulation of TNF-α bioavailability in plasma as they inactivate, to a great extent, the bio-activity of TNF [10, 11]. Also, they prolong TNF-α activity in the circulation by protecting it against degradation [4].

Several bioassays and immunoassays, including radio immunoassays (RIAs), immunoradiometric assays (IRMA) and enzyme linked immunosorbent assays (ELISAs) for the measurement of TNF-α have been described [12-20]. Most if not all these assays have some disadvantages such as lack of sensitivity or specificity, and questionable reliability. Despite the availability of many assays, TNF-α detection in the circulation therefore leads to inconsistent results [12, 19, 21, 22]. This may be due to the method of sample preparation [23, 24], the type of assay, or the presence of cytokine binding proteins in human samples [9, 25-27].

Bioassays are sensitive but have low specificity and are poorly reproducible. The disadvantages of RIAs are the need for radioisotopes and the rapid decay of the radioactive label, leading to reduced sensitivity and increased non-specific binding.

ELISAs show excellent reproducibility and high specificity, but because of steric hindrance they may leave TNF-α fragments or complexed forms undetected. Another problem in traditional ELISA methods is the interference by heterophilic antibodies. In approximately 4% of human serum samples, endogenous heterophilic antibodies are present [28, 29] which interfere with two-site ELISAs by bridging pre- and post-analyte antibodies in the absence of analyte, thereby evoking false positive signals. The interfering antibodies include human anti-animal antibodies (HAAAs) covering anti-mouse antibodies (HAMAs) and

several other types of heterophilic antibodies, including multivalent human auto-antibodies, such as rheumatoid factors.

Until recently, TNF-α concentrations were measured by means of RIA in our institute [30, 31]. The absolute values obtained by this method are higher than those of commercial ELISAs and also this assay shows no satisfying parallelism. In earlier reports from our laboratory "four-stage/two-site" ELISAs were described for highly sensitive and reproducible assessment of a number of biomarkers [32, 33]. In this format, two antibodies (coating and capture) are employed in the pre-analyte and two antibodies (trapping and detection) in the post-analyte stage. Recently, we described an ELISA format that is not susceptible to interference by heterophilic antibodies [34]. This was achieved by employing two avian (duck, chicken) antibodies in the pre-analyte and two mammalian (rabbit, goat) antibodies in the post-analyte stage.

In the present study we used such a format with the aim of developing a robust, sensitive ELISA for the assessment of TNF-α in serum/plasma samples and in cell culture supernatants, which is not susceptible to interference by heterophilic antibodies.

METHODS

Apparatus

Nunc Maxisorp™ flat-bottomed immuno-plates used in ELISA experiments were from Life Technologies (Breda, The Netherlands). The washing procedures were performed using a 96PW plate washer (SLT Lab Instruments GmbH, Salzburg, Austria). Absorbance (A) values were measured using an automated ELISA reader (Multiskan Ascent, Lab Systems, Helsinki, Finland) at 492 nm.

Reagents

For immunisation, recombinant human (rh) TNF-α was obtained from Boehringer Mannheim (Almere, The Netherlands). As standard in the ELISA rhTNF-α from Genentech Inc. (San Francisco, CA, USA) and rhTNF-α WHO reference reagent (#87/640) from the National Institute for Biological Standards and Controls (NIBSC, South Mimms, Potters Bar, UK) were used. Goat anti-rabbit IgG labelled with horseradish peroxidase (HRP) (#A-0545), bovine serum albumin (BSA) (#A-7906) and sodium perborate capsules were supplied by Sigma Chemical Co (St.Louis, MO, USA). Orthophenylenediamine (OPD) tablets (2 mg per tablet) were from Dako A/S (Glostrup, Denmark). All other reagents used were of analytical grade.

The composition of the buffers was as follows: coating buffer, 15 mmol/L Na_2CO_3 and 35 mmol/L $NaHCO_3$, pH 9.6, phosphate-buffered saline (PBS), 140 mmol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L KH_2PO_4 and 8.1 mmol/L Na_2HPO_4, pH 7.4, blocking buffer, 1% BSA in PBS, washing buffer, 0.1% Tween-20 in PBS, dilution buffer, 1% BSA in washing buffer, colour buffer, 0.05 M citrate-phosphate buffer containing 0.03% sodium perborate, pH 5.0, was made by dissolving 1 capsule sodium perborate in 100 mL aqua dest. substrate, 4 mg OPD in 11 mL colour buffer.

Polyclonal antibodies

Antibodies to chicken IgY (#14881, Sigma Aldrich Chemie, Zwijndrecht, The Netherlands) were raised in sheep and duck, whereas antibodies against rhTNF-α were raised in chicken and rabbit as described earlier [32]. The blood samples from sheep and rabbit were collected in heparin tubes, and the plasma obtained was stored at -20°C. Isolation of the IgY fraction from the chicken and from the duck egg yolk were performed as described earlier [32, 34]. Isolates were stored at -20°C. Polyclonal anti-chicken IgY and polyclonal anti-TNF-α antibodies were only used after affinity chromatography purification (Affi Gel®15, Bio-Rad Laboratories, Hercules, CA, USA) as previously described [32]. All purified antibodies were diluted with glycerol (1:1) and stored in aliquots at -20°C. Under these conditions the purified antibodies could be used for at least 12 months.

ELISA procedure

All incubation steps were separated by washing steps (4 times, 300 µL of washing buffer per well). The coating antibody was diluted in the coating buffer, whereas capture, trapping and detection antibodies, as well as standards, unknowns and reference samples were diluted in dilution buffer (100 µL per well). The procedure started with treating the microtitre plates with the coating antibody solution, *i.e.* duck-anti-chicken IgY (overnight at 4°C). In one experiment, the microtitre plate was coated with sheep anti-chicken antibodies instead of duck anti-chicken antibodies in order to check the assay for interference by HAMAs. Afterwards, the plates were blocked with the blocking buffer (300 µL per well, 2 h at 37°C). The next step was the incubation with the capture antibodies, *i.e.* chicken anti-TNF-α (2 h at 37°C). The incubation with the standards, unknowns and reference samples took place overnight at 4°C. The incubation with trapping antibody (rabbit anti-TNF-α), as well as the subsequently incubation with detection antibody (HRP-labelled goat anti-rabbit antibody, dilution 20,000 times) were performed over 2 h at ambient temperature. The incubation with substrate solution was performed in darkness for 60 minutes at ambient temperature. When the colour reaction was completed, 100 µL of 1 M H_2SO_4 per well was added to stop the reaction. The absorbance values were measured at 492 nm within 30 min.

Serum, plasma and control preparations

As sampling conditions can affect the measurement, strict precautions were taken during sampling to avoid blood cell stimulation, which would have falsely increased TNF-α levels [24, 35]. Immediately after collection, blood tubes were placed on melting ice and centrifuged at 4°C to avoid *ex vivo* cytokine production.

Plasma (EDTA or heparin) samples were routine patient samples collected at our department and stored at -80°C until analyses. Whole blood was collected from healthy persons, in three 2 mL vacutainer tubes (Li heparin, Becton and Dickinson #367681). One tube was used to measure circulating TNF. The other two tubes were used to measure *ex vivo* TNF production. To one tube 10 ng/mL LPS (lipopolysaccharide *E.coli* serotype 055:b5, Sigma)

was added and both tubes (+LPS and -LPS) were incubated for 6 h at 37°C. Blood was centrifuged at 2100g for 10 min and the plasma separated. An additional centrifugation step was performed at 14,000 *g* for 5 min in order to completely remove platelets. All samples were diluted 5 times in dilution buffer before use.

To determine the intra-assay precision 80 plasma samples were tested. For parallelism experiments, heparinised blood (Li heparin, Becton and Dickinson) and supernatants of cultured peripheral blood polymorphonuclear (PBMNC) cells were used. Twenty-five serum samples from individuals with human anti-mouse antibodies (HAMA) were supplied by Scantibodies Laboratory Inc, Santee, USA (code: P9825/148-P11906/194, HAMA concentrations were established by Scantibodies HAMA Assay (IRM:3KJ002) and ranged from 42-813 ng/mL).

To assess the ability of the assay to avoid interference by heterophilic antibodies, two plasma samples positive and two negative for rheumatoid factor, were spiked with either 0.84 or 2.4 ng of recombinant TNF-α.

In each run, two control samples were assayed to check between-assay variability and to monitor overall long-term performance of the assays. For the first control sample (denoted No. 5), EDTA plasma from a healthy individual stimulated with LPS was aliquoted and stored at -20°C. The second preparation, marked 230500, was prepared by pooling 2 plasma samples (EDTA-plasma and EDTA-plasma stimulated with LPS over 6 h at ambient temperature, respectively). The pool preparation was aliquoted (50 µL/vial) and lyophilised. Prior to the assay, Pool 5 was thawed and diluted 5 times with dilution buffer, whereas control preparation 230500 was reconstituted in 0.25 mL dilution buffer.

For the comparison of the ELISA with the in-house RIA, plasma from patients with tuberculosis (n = 19) and plasma from healthy controls (n = 8) were used. In addition, the performance of two commercial ELISA kits were tested, using buffer spiked with a series of concentrations of the WHO standard for TNF-α.

RIA procedure

The "in-house" radioimmunoassay (RIA) for TNF-α used was described in detail earlier [30, 31]. The recovery of TNF-α in our RIA was estimated at 93%. However, no satisfactory parallelism was achieved with this assay. To limit this problem, fixed sample volumes were used for measurement of circulating TNF-α (100 µL) and *ex vivo* production (25 µL).

Neutralisation of bioactivity of TNF by the anti-TNF antibody

To find out whether the polyclonal IgY antibody neutralises TNF-α we isolated human peripheral blood mononuclear cells from 2 healthy donors by density centrifugation over a Ficoll gradient (Ficoll-Pacque, Amersham #17-1440-03), and cultured these in RPMI (1640 Dutch mod., Gibco #22409-015) for 24 hours as described in detail elsewhere [30]. The cells were incubated with or without 5 µg/mL of TNF-α, and with or without the IgY antibody against TNF (in concentrations of 1.8 µg/mL). As a readout for the biological effect of TNF-α, we measured the concentrations of interleukin-6 in the supernatants by ELISA, according to the manufacturer's instructions (Sanquin Pelikine kit #M1916).

Statistics

All measurements were performed in duplicate.

The standard curves were approximated as polynomials $Y=a+bX+cX^2$ in which *X* stands for an analyte concentration and *Y* stands for absorbance value. The polynomial coefficients *a*, *b* and *c* were calculated by the method of least squares. Detection limits were calculated at the 95% level of confidence on the basis of general variance of experimental data and the slopes of the curves. The agreement between experimental data was evaluated with the Pearson correlation or the Spearman rank correlation test (r_s), whenever appropriate. Difference plots according to Bland and Altman [36] were made. Regression analysis according to the method of Passing and Bablok [37] was used. Two-sided p-values below 0.05 were considered to be statistically significant.

RESULTS

Standard curves, analytical and functional sensitivity

The dose-response curves for the two TNF-α standards are shown in *figure 1A*. The relative potency of the recombinant human TNF-α (Boehringer) standard and the NIBSC rhTNF standard, were similar. The analytical sensitivity of the TNF-α ELISA, defined as the dose of analyte producing a response greater than two standard deviations above blank values, was found to be 8 ± 4 pg/mL for both standard curves. The functional sensitivity i.e. the amount of TNF-α that could be measured with a precision of at least 20% was 13 pg/mL.

Precision and stability

The within-assay precision was assessed by plotting the coefficient of variance (CV) of duplicate measurements of plasma against determined TNF-α concentrations. For plasma samples (n = 80) the variation differed from approximately 20% at 13 pg/mL to 5% at higher (> 100 pg/mL) levels of TNF-α *(figure 1B)*. The between-assay precision was assessed by analyzing in each assay run two control samples, 230500 (n = 22) and Pool 5 (n = 24), during almost one year of TNF-α measurements. No significant changes in TNF-α concentrations were observed during this period in these samples. For the control preparation 230500 the obtained TNF-α level (mean ± SD) was found to be 0.81 ± 0.09 ng/mL with an inter-assay precision of 11.1%. In the control preparation Pool 5 TNF-α level in this period was 0.22 ± 0.02 ng/mL (mean ± SD) with an inter-assay precision of 9.6%.

Recovery and parallelism

Recovery of TNF-α was studied by adding known TNF quantities to three different EDTA plasma samples *(figure 2A)*. The endogenous concentrations of TNF-α in these plasma samples were 8, 14 and 54 pg/mL, respectively. In these samples the recovery of 250 pg/mL, 500

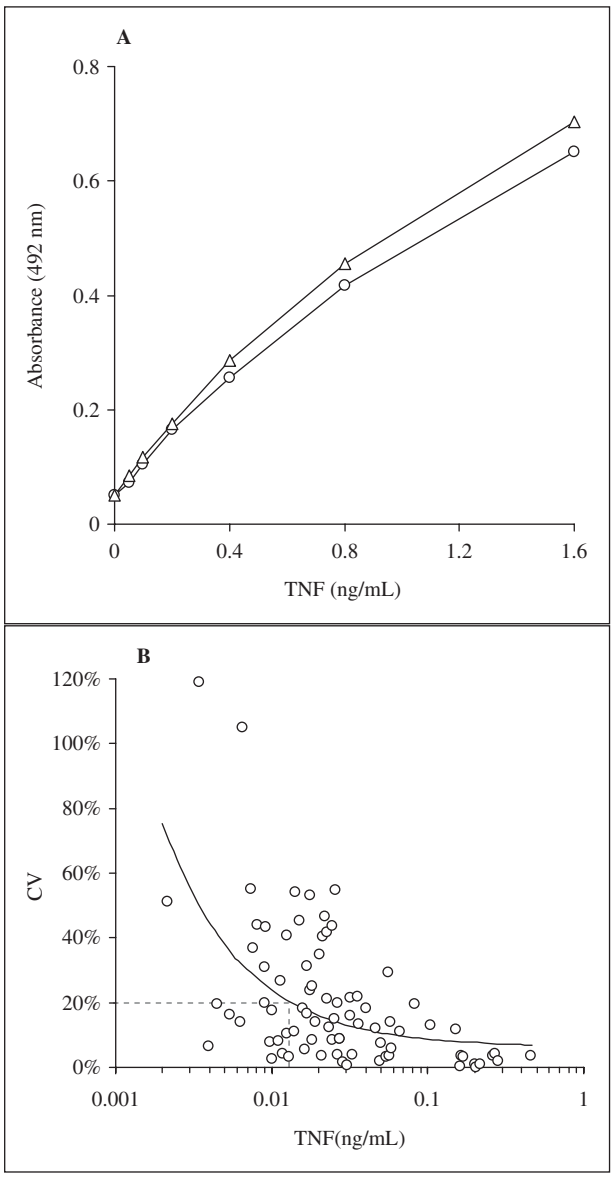

Figure 1

A) The dose-response curves of two TNF-α standards; recombinant human TNF-α from Boehringer (-○-) and the WHO reference standard from the NIBSC (-Δ-). **B**) Precision profile for TNF-α ELISA. CVs of duplicate measurements of 80 EDTA plasma samples are plotted against TNF-α concentrations found directly in wells, before being corrected for the dilution factor. The functional sensitivity of the assay is the analyte concentration that corresponds to 20% CV of the precision profile.

pg/mL and 1000 pg/mL added TNF-α was 119 ± 7% (mean ± SD), 99 ± 11% and 97 ± 9%, respectively *(figure 2A)*.

To study the parallelism, plasma samples (n = 5) and PB-MNC supernatants (n = 5) were diluted 6, 12, 24 and 48 times in dilution buffer. As can be observed in *figure 2B* and *2C* parallelism was excellent in plasma samples as well as in cell culture supernatants.

Comparison of the new ELISA with the "in-house" RIA and with commercial ELISAs

Values obtained by the new ELISA assay were compared with those obtained by the "in-house" RIA and with two commercially available ELISAs. For the comparison between the RIA and the new ELISA, plasma samples

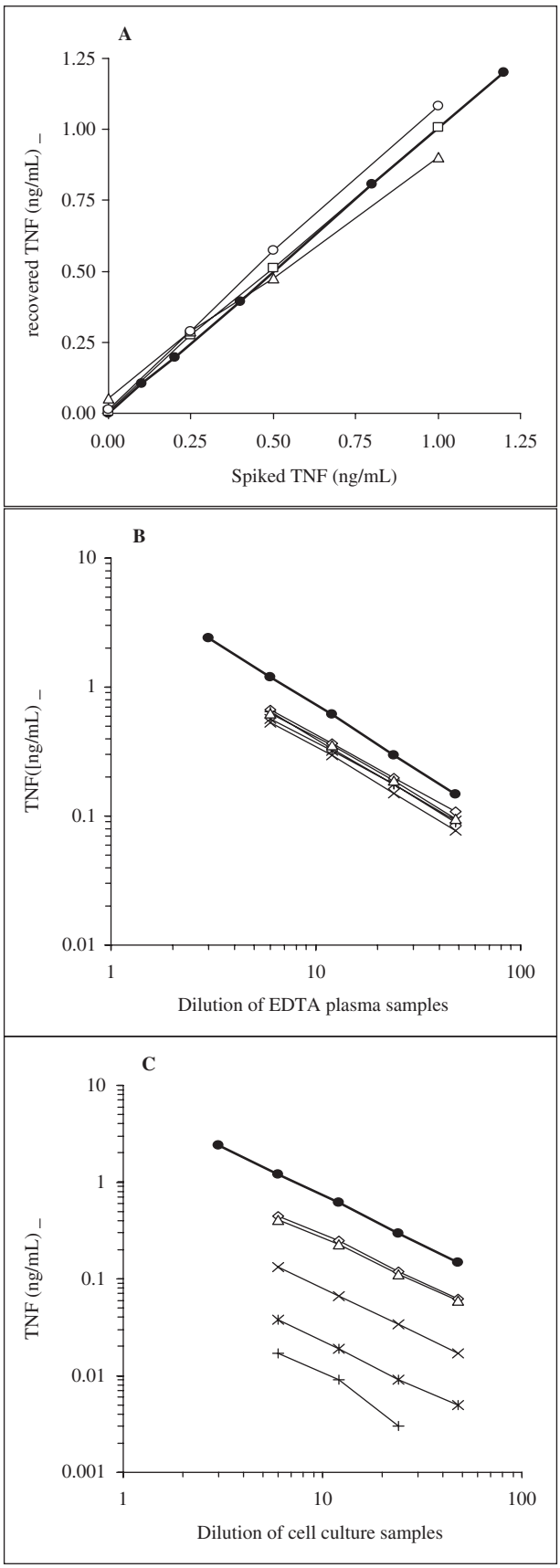

Figure 2

A) TNF-α recovery. Three EDTA plasma samples were spiked with TNF-α (250, 500 and 1000 pg/mL, thin lines). For comparison, the standard line of pure calibrator is shown (thick line). **B** and **C**) Parallelism. Serial dilutions (6, 12, 24 and 48 times) of EDTA plasma samples (**B**) and cell culture supernatants (**C**) (thin lines). Serial dilutions of standards are shown as thick lines (black spots).

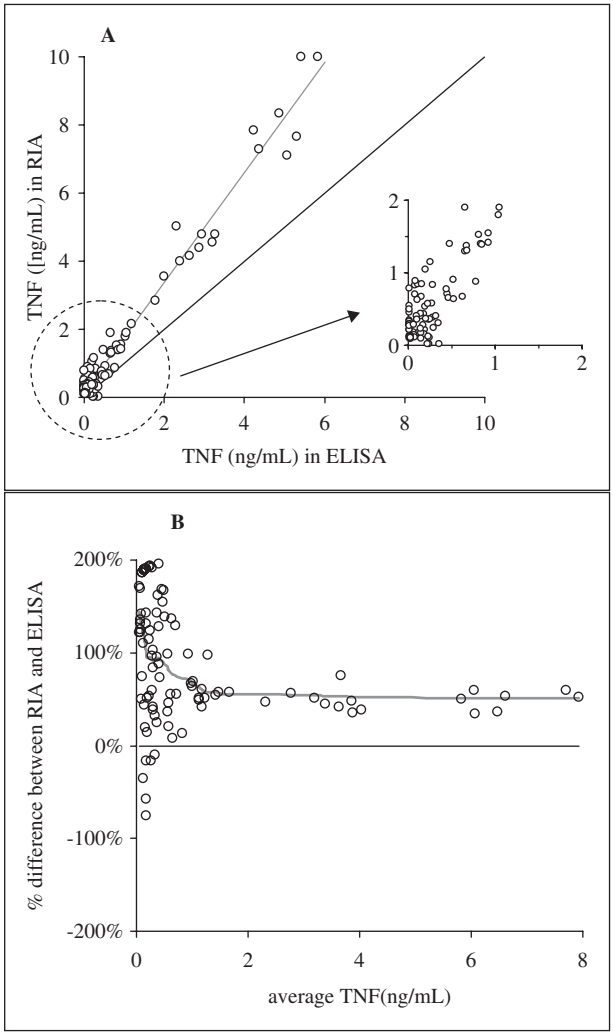

Figure 3
A) Relationship between the TNF-α ELISA and the "in-house" RIA. The dotted line represents the line of equality, whereas the Passing-Bablok regression line is shown as the solid line. The area of low TNF-α concentrations is highlighted in the insert. **B**) Difference plot, demonstrating the agreement between the TNF-α ELISA and the "in-house" RIA. Above the level of 1 ng/mL, the value of relative difference between "in-house" RIA and our TNF-α assay is approximately 50%. Below 1 ng/mL, the differences between TNF-α values assessed by RIA and those obtained by ELISA are higher.

(n = 97) collected for routine laboratory investigations were taken. The TNF-α preparation from Boehringer was used as a calibrator in these assays. The TNF-α concentrations obtained by "in-house" RIA (range: 0.095-10.0, median 0.578 ng/mL) were found to be higher than obtained with the ELISA (range 0.008-5.84, median 0.213 ng/mL) *(figure 3A)*. Spearman correlation was 0.755 ($p < 0.0001$). Using regression analysis, an intercept of 0.104 ng/mL was found, whereas the slope was 1.66 ± 0.10 *(figure 3*A*)*. The Bland Altman difference plot of these data clearly confirmed that the ELISA produces lower TNF-α values than the RIA *(figure 3B)*. In particular, at low concentrations (below 1.0 ng/mL) the RIA tended to yield even more elevated TNF-α values than ELISA.

For the comparison with the commercial ELISAs the WHO TNF standard was used. The results of these experiments are presented in *table 1*. As can be seen from this table, the commercial ELISAs have a rather poor precision, they either greatly overestimate or underestimate the concentration of the WHO standard. Our in-house RIA and the new ELISA have an excellent performance. In our hands the claimed sensitivity of one of the ELISAs was not found. The sensitivity of our new ELISA appears to be in the same range as that found for the commercial ELISAs. It is clear that the RIA is less sensitive than the ELISAs.

Interference of heterophilic antibodies

To check whether interferences of heterophilic antibodies occur in the "duck" ELISA (in which the coating antibody was raised in a duck against chicken IgY), TNF-α was measured in 25 HAMA containing sera. In parallel, TNF-α in these samples were also analysed in a "sheep version" of the assay (the coating antibody being raised in a sheep against chicken IgY). In the "sheep version" of the ELISA, mammalian antibodies were consequently present at both pre- and post-analyte stages, an assay structure that potentially is vulnerable to interference by heterophilic antibodies present in blood [34]. In the present "duck version" only avian antibodies are employed in the pre-analyte stage and only mammalian antibodies in the post-analyte stage. In *figure 4* the HAMA concentration is plotted against the TNF-α concentration obtained in 25 HAMA containing sera. As can be observed in the "duck version" *(figure 4A)* TNF-α concentrations in these sera were low, 11 out of 25 were not detectable (< 0.08 ng/mL). The other 14 sera ranged from 0.088 to 0.534 ng/mL. In the "sheep version", however *(figure 4B)*, considerable concentrations of TNF-α were measured (ranging 0.141 to 15.43 ng/mL), whereas in only 4 out of 25 samples TNF-α was not detectable.

In addition we assessed the ability of the assay to avoid interference by rheumatoid factor. In two plasma samples containing rheumatoid factor and spiked with recombinant TNF-α we found a mean recovery of TNF-α of 104%

Table 1
Comparison of the new, 4-span ELISA with 2 commercial ELISAs and RIA

WHO standard	4 span ELISA	ELISA 1	ELISA2	RIA
	pg/mL	pg/mL	pg/mL	pg/mL
1000 pg/mL	1090	375	1558	1093
250 pg/mL	278	87	388	251
Sensitivity claimed by manufacturer	-	1	none	-
Sensitivity found	8 resp 13[a]	3	16	40

[a] 8 is mean calculated zero signal + 2 SD, 13 is functional sensitivity.

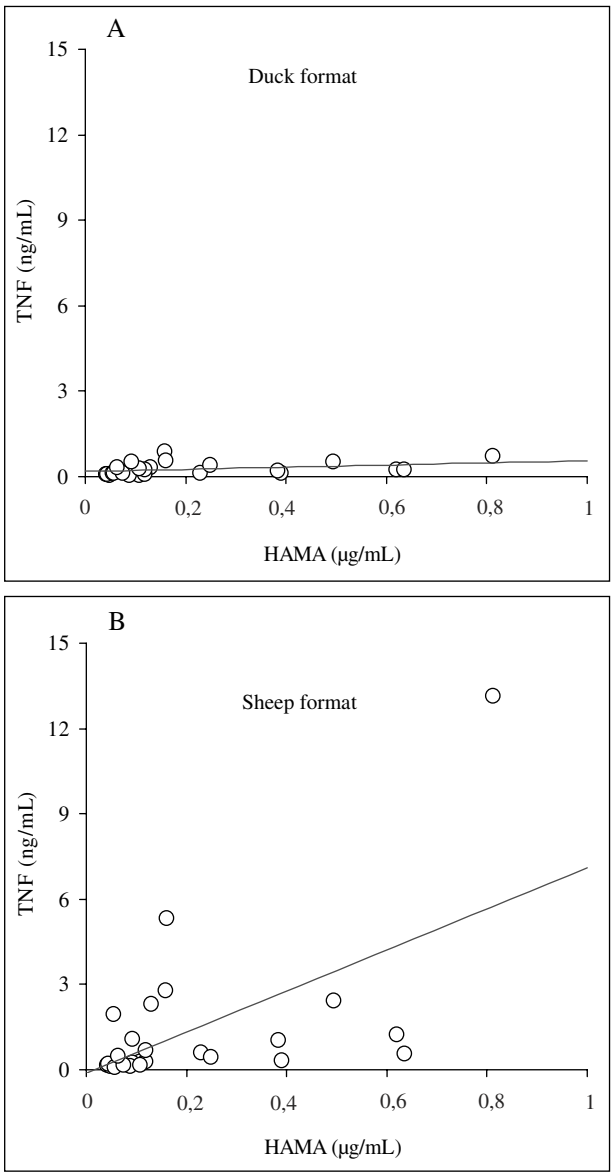

Figure 4
Antigen values obtained with TNF-α assay in the duck format (**A**) and in the sheep (**B**) format in 25 samples containing different concentrations of HAMAs. Clearly, the sheep format is affected by the heterophilic antibodies.

(range 94-120%). In control plasma without rheumatoid factor the recovery was very similar (mean 100%, range 88-112%)

TNF-α concentrations in TB patients and healthy persons

In order to investigate whether the new ELISA could discern TNF-α concentrations between control and diseased persons, plasma samples from healthy individuals and from TB patients were assayed for TNF values using the ELISA as well as the RIA. *Figure 5* shows that the concentrations of TNF-α obtained with the RIA for the healthy subjects (range: 0.10-0.14 ng/mL, median 0.12 ng/mL) were significantly lower ($p < 0.0001$) than those obtained from the TB patients (range: 0.25-1.42 ng/mL, median 0.41 ng/mL). Also the concentrations of TNF-α obtained with the ELISA in the plasma from the healthy individuals (range: 0.04-0.08, median 0.04 ng/mL) were

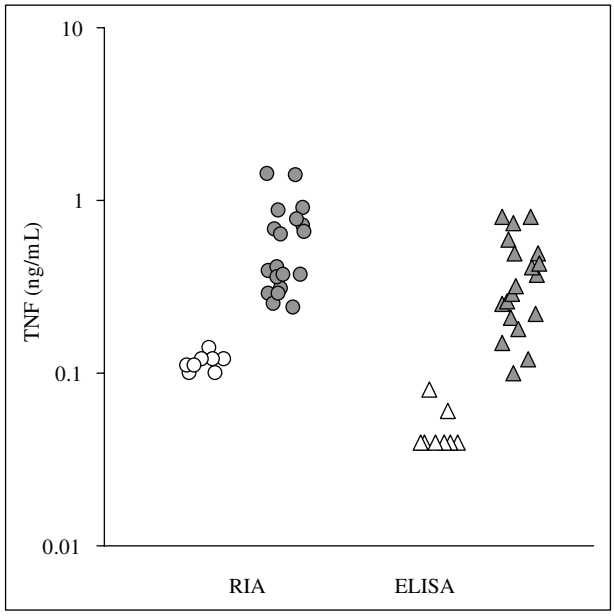

Figure 5
Comparison of the results obtained for the plasma samples from healthy individuals (n = 8) using the "in-house" RIA (-Δ-) and our TNF-α ELISA (-○-) with those for TB patients (n = 19).

significantly lower that those in plasma from TB patients (range: 0.81-0.10, median 0.32 ng/mL) ($p < 0.0001$). In general, the concentrations obtained with the ELISA were lower than those obtained with the RIA.

Neutralisation of TNF-α by the IgY anti-TNF-α antibody

At concentrations of 1.8 µg/mL the chicken anti-human TNF-α antibody appeared to block IL-6 production of cultured peripheral blood mononuclear cells stimulated with TNF-α.

DISCUSSION

In the present study, we describe an ELISA for TNF-α based on the format earlier developed for analysing components of the plasminogen activator system and VEGF [32-34]. The four-antibody sandwich ELISA format is multi-applicable, as the exchange of the specific capture and trapping antibodies by polyclonal antibodies raised against TNF-α in chicken and rabbit lead to an assay that is sensitive, robust, shows good parallelism and, particularly important when used in patients, is not susceptible to interference by heterophilic antibodies. The assay therefore is an improved tool for measuring both low TNF-α concentrations in healthy individuals and elevated concentrations in patients.

During the last decades, several assays for TNF-α have been described, all with their advantages and disadvantages. Although bioassays can be more sensitive than immunoassays, they can be affected by a multitude of endogenous and environmental factors. RIAs mostly offer more specificity, but reduced sensitivity. A rapid deterioration of tracer could result in high, non-specific binding and thereby, reduced sensitivity. However, using freshly prepared label could circumvent this [23]. Several methods have appeared in the literature for the measurement of

TNF-α by conventional ELISA [19, 38-40]. An inherent problem of these assays is that different test kits may generate different test results, mainly because of differences in the antibody specificity and/or affinity used in different test kits and because of the use of different standards and buffers provided with the kits. Obviously, one cannot assume that one method for the assessment of TNF-α present in blood will provide the same results as another method. Clearly, the techniques used must be appropriate, validated and evaluated extensively. This also includes pre-analytical conditions [23, 41].

As TNF-α in blood occurs in different molecular forms, the discrepancies between different assays might be related to the presence in the biological fluids of antigenic forms of TNF-α that are not detectable by some assays [19, 42]. Use of polyclonal antibodies does not necessarily mean that biologically active TNF is being detected. Our polyclonal IgY antibody does neutralise TNF activity, as was shown by inhibition of IL-6 induction. Thus, although the antibody can be used for a neutralisation assay, obviously this finding does not imply that the ELISA detects just bioactive TNF-α.

The performance of different assays is further affected by the interaction of TNF-α with various plasma components such as TNF-α binding proteins, comprising soluble TNF receptors [19], autoantibodies [43] and natural occurring serum proteins such as β2-macroglobulin [26]. These TNF-α binding substances may have an unpredictable influence on TNF-α levels measured, and its concentration may change with inflammatory or infectious diseases. Complex dissociation and differential changes in the TNF-sTNF-R1 bound-free ratio in different assays for instance, markedly affects TNF-α quantification [20]. In addition, the dissociation between TNF-α and sTNF-R is relatively rapid and the ratio bound:free in samples may even change during assay incubations *in vitro* [20].

The values obtained with our RIA in blood from patients with TB are higher than those obtained with our ELISA. In particular, a clear trend was observed for low TNF-α concentrations (below 1.0 ng/mL). Our competition radioimmunoassay [31] used an unpurified, rabbit polyclonal antiserum against TNF-α. It is known that polyclonal antibodies contain antibodies for several epitopes and thus can probably detect different forms of TNF-α, subunits, TNF-α complexed with receptor, and degradation products. Our TNF-α RIA does indeed detect both free and soluble receptor-bound TNF-α [44]. The capability of our new ELISA to detect free TNF-α or the receptor-bound TNF-α has to be worked out, but in general ELISAs are more specific than RIAs. It is known that contaminants in unpurified antibodies can interfere in RIAs in which antibody-tracer complexes are separated from the free label by use of a second antibody, leading to falsely elevated assay results. The polyclonal antibodies used in the ELISA are affinity purified and therefore largely depleted from such contaminants. In the RIA, only one polyclonal antibody is used, whereas in an ELISA two polyclonal antibodies sandwich the TNF-α molecules. The lower TNF-α levels obtained in our ELISA could consequently result from steric hindrance due to immobilisation of the capture antibody or, more likely, from higher specificity for free TNF-α.

The analytical sensitivity of the ELISA, 8 pg/mL, compares favourably with our RIA (20 pg/mL [30]) and is in the same order as commercial ELISAs for TNF-α found in this paper and as described earlier [15]. In this ELISA, the potency of the Boehringer recombinant human TNF-α is almost identical with the NIBSC/WHO First International Standard. The recovery of TNF-α in our RIA was estimated at 93%, but no satisfactory parallelism was achieved. To limit this problem, fixed sample volumes were used for measurement of circulating TNF-α [30], which obviously is not satisfactory. Dilution experiments show a good parallelism for serum as well as cell culture-medium supernatants in the ELISA, indicating that both recombinant and native TNF-α present in biological samples are equally recognised by the assay.

Although TNF-α is known as a central mediator in the pathogenesis of inflammation, and its role in pathology has been well documented, assay results may be quite heterogeneous with different assays and may produce conflicting results about the role of TNF-α. A frequent problem encountered in ELISA is the susceptibility of the assay to substances that might interfere with the assay, such as rheumatoid factor, human-anti-mouse antibodies (HAMAs) or heterophilic antibodies. These antibodies appear frequently in patient samples, and lead to false-positive values by aspecific cross-linking of pre- and post-analyte antibodies. The use of avian antibodies in the pre-analyte stage and mammalian antibodies in the post-analyte stage lead to an assay concept that shows no false-positives by HAMA interference or rheumatoid factor [34]. When analysing a set of 25 HAMA-positive serum samples, clear false positive signals were obtained in the "sheep version" of the TNF ELISA. Analyzing these samples in the TNF ELISA version, with only avian antibodies at the pre-analyte stage, very low concentrations of TNF were measured, indicating that this format is not susceptible to interference by heterophilic antibodies

To obtain a greater understanding of the role of TNF-α, and because of the increasing interest in the metabolic, non-immune roles of this pleiotropic cytokine, it is important to be able to measure accurately its levels in different disease states. It is therefore essential to have available, a robust, sensitive and reliable assay, like the present one.

Acknowledgements. *The TNF ELISA described in the present paper has been developed by the Dept. of Chemical Endocrinology and General Internal Medicine, and is available for research purposes. For reagents and questions about this kit contact Pr. Fred C.G.J. Sweep, Dept of Chemical Endocrinology 530, Radboud University Nijmegen Medical Center, Geert Grooteplein 8, 6525 Nijmegen, The Netherlands. <f.sweep@ace.umcn.nl>*

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