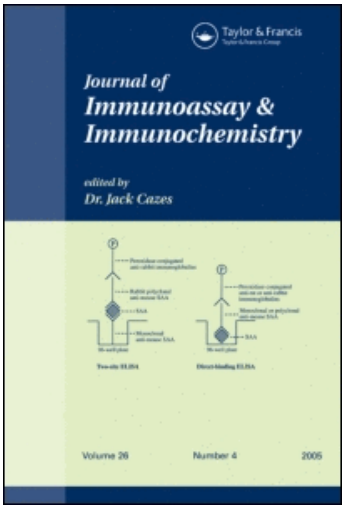

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

A New Efficient Method for Eliminating the Interference Effect of Human Serum and Increasing the Sensitivity and Recovery Rate of Enzyme Immunoassay

Resul Karakus[a]; Bugra A. Buyrukcu[a]; Cemalettin Aybay[a]
[a] Department of Immunology, Faculty of Medicine, Gazi University, Besevler, Ankara, Turkey

To cite this Article Karakus, Resul , Buyrukcu, Bugra A. and Aybay, Cemalettin(2005) 'A New Efficient Method for Eliminating the Interference Effect of Human Serum and Increasing the Sensitivity and Recovery Rate of Enzyme Immunoassay', Journal of Immunoassay and Immunochemistry, 26: 2, 109 — 124
To link to this Article: DOI: 10.1081/IAS-200051994
URL: http://dx.doi.org/10.1081/IAS-200051994

Journal of Immunoassay & Immunochemistry, 26: 109–124, 2005

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1081/IAS-200051994

A New Efficient Method for Eliminating the Interference Effect of Human Serum and Increasing the Sensitivity and Recovery Rate of Enzyme Immunoassay

Resul Karakus, Bugra A. Buyrukcu, and Cemalettin Aybay
Department of Immunology, Faculty of Medicine, Gazi University, Besevler, Ankara, Turkey

Abstract: A new, very simple method for increasing the sensitivity and recovery rate of enzyme-linked immunosorbent assay (ELISA) for the precise quantification of antigen in human serum is described. The assay design uses CATNF6A4c IgG2a monoclonal antibody and biotinylated anti-human tumor necrosis factor-α (hTNF-α) polyclonal mouse IgG as the capture and tracer antibodies, respectively. The assay is completed within 4 hours at room temperature and is capable of detecting both recombinant and native human TNF-α.

The assay incorporates the use of saturated ammonium sulfate (SAS) as a component of the dilution buffer to amplify the resultant signal from antigen containing human serum and eliminating the endogenous interference of native human serum. SAS worked optimally at the final concentrations, ranging from 1.2% to 11%. The addition of SAS to the dilution buffer resulted in a dramatic increase in both sensitivity and recovery rate of the ELISA.

The results demonstrated that 50 μL of dilution buffer, containing SAS, enabled the precise quantification of human TNF-α in 100 μL of human serum samples and eliminated the interference of native serum, which seemed to be related to complement proteins. Therefore, dilution buffer containing SAS, at a defined concentration, seemed to be a potential candidate for resolving sensitivity and recovery problems usually encountered in immunoassays when measurement was performed with native

Address correspondence to Resul Karakus, Department of Immunology, Faculty of Medicine, Gazi University, Besevler 06500, Ankara, Turkey. E-mail: resul_karakus@yahoo.com

serum samples. The proposed technique provides an easy, practical, and consistent method for ELISA when using human native serum.

Keywords: ELISA, Sensitivity, Recovery rate, Serum, Complement, Interference, Ammonium sulphate, Human tumour necrosis factor alpha

INTRODUCTION

The increasing availability and use of immunoassays in research, as well as in clinical laboratories, has mounted a number of problems.[1,2] One of the major problems confronted in immunoassays is that the overall recognition between the analyte and the reagent is restricted to a limited portion of the molecule. Another difficulty is characterized by the fact that these methods operate at very low concentrations and are very sensitive to the enviromental conditions created by molecules present in much higher concentrations. These factors hinder achieving good analytical performance in immunoassays.[1,3–6] It has been shown that different sample matrices significantly affect the functional sensitivity, precision, and dilution linearity of certain immunoassays designed for particular molecules.[4]

Interference observed in immunoassays is defined as interference from human antibodies which are of sufficient titer and affinity to have an analytically significant effect and the immunogen yet to be identified.[7] Interference resulting from the use of human serum has an insidious and unpredictable nature.[8] Heterophilic antibodies, such as human anti-mouse antibodies (HAMA), have an important impact on interference, especially in solid-phase sandwich immunoassays based on monoclonal antibodies. Rheumatoid factors, though their affinity is usually too low to cause significant interference, are also being regarded as factors for interference in immunoassays.[9,10] In general, apart from the nature of the reagent and the analyte, interference may lead to falsely elevated or depressed results, which may mean wrong decisions based on these results. For reasons mentioned above, and for several other interference factors, it is being recommended that special attention should be given to the HAMA issue and other interfering factors.[11] Although there is no guarentee that any given assay will not be effected by HAMA, sample pretreatment, use of Fc-removed capture antibodies, or chimeric antibodies, heat-pretreatment has been addressed to remove or minimize the potential effects of interfering factors, including HAMA.[7,11–14]

The material often recommended for immunological assays is native serum. A freshly drawn serum sample contains an active complement system, which might not be sufficiently inactivated, even during storage. The complement system is a potent amplification system in humoral immunity. The split-product, C3b, binds to immune complexes formed during an immune reaction and acts as an opsonin. There are also counter balancing factors, such as

factor H and I, which promote the degradation of the active C3b molecules. Because mammalian antibodies used in most immunological assays may activate the human complement system, and these active components could bind to antibodies,[15] there is always a potential risk of partly or totally blocked antibody binding epitopes when native human serum is used in immunoassays without pretreatment or any other interference preventive measures.[16] Several assays have clearly demonstrated this phenomenon.[15,17–19]

In order to overcome interference related issues resulting from the use of human serum, assays have been performed with the use of antibodies originating from several other species, i.e., other than mouse. In this regard, isotypes of mouse or human immunoglobulins have also been found to be responsible for the degree of interference in immunoassays. Human IgG1 and G3, as well as mouse IgG2a and G2b, are found to be strong activators of the complement proteins.[19] Chicken IgG was found to not activate the human complement system and, therefore, it is recommended to be used in solid-phase immunometric assays to reduce interference.[18,19] Although antibodies derived from egg yolk seemed to be offering many advantages over mammalian antibodies in several aspects, most of the antibodies used in this research, as well as in the diagnostics area, are of mouse origin and,therefore, this kind of approach seems to be not practical. Since affinity and, in part, the isotype of the monoclonal antibodies, are determined randomly, it is not always possible to have a high affinity immunoglobulin (Ig) that would be preferable, due to their low activation potential in regard to the complement system. Hence, there should be at least a "plan B" for overcoming assay related troubles, such as interference originating from the sample itself.[17,18]

Another method for overcoming interfering factors in human serum would be to precipitate the factors, i.e., proteins, but the analyte itself. In a study using a modified "two-site" immunoradiometric assay, the occurrence of non-analyte antibody-binding substances has been found in about 40% of the serum samples. Because these antibody-binding substances mimic the presence of analyte, the possibility of over- or under-estimates of the analyte is regarded as a potential risk with the immunoassay.[20]

In the case of human serum, several precipitation agents have been used for a long time to purify certain molecules. Although this may denature some monoclonal antibodies, it is well known that saturated ammonium sulfate (SAS), at certain concentrations, precipitates Ig's, and this method is still being used in Ig purification in conjuction with others following more sophisticated and discriminating methods. Because SAS is highly soluble in water, it stabilizes most proteins in solution, favors protein interactions rather than protein-solvent interactions, promotes aggregation of proteins, and helps reduce the lipid content of the sample. Since each protein has a different solubility, this method is useful for isolating groups of proteins, as well. These may account for the pros and cons of precipitation with SAS.[21,22] At certain concentrations of SAS, specific protein groups are precipitated from normal human serum.[23,24]

From this point of view, we investigated the possible roles of SAS in preventing the so-called non-specific interference factors, especially those that may be attributable to complement proteins found in native human serum, on the measurement of TNF-α using ELISA.

EXPERIMENTAL

Reagents

The following materials were purchased from commercial suppliers: RPMI-1640, L-glutamine, Ficoll-Hypaque, lipopolysaccharide (LPS) from *Salmonella thyphimurium*, complete Freund's adjuvant, incomplete Freund's adjuvant, ethylene diamine-tetra acetic acid (EDTA) (Sigma, St. Louis, MO, USA); Foetal calf serum (FCS; FCS was heat-inactivated by keeping at 56°C for 30 minutes), Bovine serum albumin (BSA; Fatty acid free) (PAA Laboratories GmbH, Linz, Austria); EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Inc., Rockford, IL 61105); Streptavidine-Horseradish peroxidase (Vector Laboratories Inc., Burlingame, CA 94010); Recombinant human TNF-α (rhTNF-α; PeproTech, Inc., Rocky Hill NJ); Ammonium sulfate ($(NH_4)_2SO_4$) (Merck, D-6100 Darmstadt, Germany).

Cell Line

Anti-human TNF-α monoclonal antibody (mAb) producing hybridomas were established by the conventional polyethylene glycol (PEG) fusion method, using splenocytes from an immune BALB/c mouse and the NSO cells as the myeloma partner. Determination of isotype was performed with a commercially available isotyping kit (ISO-1, ImmunoType Mouse Antibody Isotyping Kit, Sigma Chem. Co., MO, USA). Among the nine different anti-human TNF-α mAb-secreting hybridomas, CATNF6A4c (isotype IgG2a, specific for both native and recombinant human TNF-α) was selected for capture antibody according to data obtained from preliminary assays.

Monoclonal Antibody Purification

Anti-human TNF-α mAb was purified from the supernatant of CATNF6A4c hybridoma cultured in RPMI-1640 supplemented with 10% IgG-depleted FCS using Hi-Trap Protein G column (1 mL MAbTrap G II Kit, Supelco Bellefonte, PA) attached to a high-pressure liquid chromatography (HPLC) system (Agilent 1100 Series HPLC system. Agilent Technologies, GmbH, Waldbronn, Germany). Protein G-bound antibodies were eluted with 0.1 M

citric acid pH 2.5 and then immediately neutralized with trizma. The eluted peak fraction was passed through a Sephadex G-25 Fine column (1×30 cm). Protein concentration was measured spectrophotometrically using bovine serum albumin (BSA) as a standard.

Production of Polyclonal Antibody

Mouse antiserum to human TNF-α was prepared by immunizing a ten-week old BALB/c mouse four times at four-week intervals. Ten micrograms of rhTNF-α was used for each injection. Recombinant hTNF-α was emulsified with complete Freund's adjuvant at 1 : 1 ratio (v/v) and injected intraperitoneally plus subcutaneously at four different sites in the abdominal region. Second and third injections of the antigen were given, after mixing with incomplete Freund's adjuvant, via the same route. Five days after the last injection of antigen in PBS only, a mouse was driven into general anaesthesia with ketamine/xylazine cocktail and then 1 mL of blood was collected; then, the serum was recovered. The whole IgG fraction containing polyclonal antibodies (pAbs) was purified from serum using a Hi-Trap protein G column.

Biotinylation of Polyclonal Mouse IgG

Biotinylation of anti-human TNF-α pAbs was performed using EZ-Link Sulfo-NHS-LC-Biotin according to the manufacturers instructions. Free biotin was chromatographically eliminated using a Sephadex G-25 Fine-packed 1×30 cm column.

Production of Native Human TNF-α

Heparinized human peripheral blood was collected. Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll-Hypaque centrifugation. Cells were washed three times with RPMI-1640 and then used for experiments. These cells were cultured at 1×10^6 cells/mL per well in 24-weII tissue culture plates (Costar. Corning Incorporated. Corning. NY. USA) containing RPMI-1640 medium supplemented with 10% FCS and with or without the presence of 0.1 μg/mL of LPS at 37°C in a 5% CO_2 atmosphere. Supernatant samples were taken from cell cultures 24 h later and used as the source of native hTNF-α.

Human Serum and Plasma

Human blood was obtained from either the laboratory personnel or the routine immunology laboratory. Human serum was separated from the coagulated

blood samples and used in the experiments described in this study. For heparinized and citrated plasma, 5 mL blood samples were drawn from each individual directly into tubes containing either 100 μL of 5000 IU/mL heparin sodium or 3.8% sodium citrate, separately. All samples were assayed simultaneously, just thereafter, without storage.

Enzyme-Linked Immunosorbent Assay (ELISA)

High binding capacity ELISA plates (Costar, No: 3590. Corning lncorporated, Corning, NY, USA) were coated with 100 μL of CAyTNF6A4c mAb at 5 μg/mL in 0.05 M carbonate-bicarbonate buffer (CBB), pH 9.6, by incubating overnight at +4°C. After washing 3 times with a washing buffer consisting of PBS plus 0.04% Tween 20 (PBS-T), plates were blocked with 200 μL of PBS-1% BSA and then incubated at room temperature (RT) for 2 hours. The plates were then washed five times with PBS-T. 100 μL of the standards or the samples were added to the wells, which contained 50 μl of dilution buffer, with or without various concentrations of SAS or EDTA. The dilution buffer consisted of 1% BSA-PBS. Plates were incubated for 2 hours at RT. After washing three times with PBS-T, 100 μL of biotinylated polyclonal antibody at 1 μg/mL was pipetted into each well and then incubated for 60 min at RT. After washing three times with PBS-T, to each well, there was added 100 μL of streptavidine-horseradish peroxidase at 0.4 μg/mL, followed by incubation for 60 min at RT. After washing 3 times with PBS-T, the reaction was revealed with 100 μL of 3,3′,5,5′ tetramethylbenzidine (TMB) solution for 30 min at room temperature. After stopping the reaction with 50 μL of 1M H_2SO_4, the optical density (OD) of each well was measured with an ELISA reader (Sunrise Elisa Reader, Tecan Austria GmbH, Grödig/Salzburg, Austria) at 450/620 nm.

Statistical Analysis

Statistical analyses were performed by Student's *t*-test. Differences were considered statistically significant if $p < 0.05$. Regression-correlation analysis was performed. Inter- or intra-assay coefficients of variation (CV) were calculated and used for the quality control of the human TNF-α ELISA. Calculations were performed with SPSS for Windows, version 10.0.

RESULTS

Effect of SAS on Human TNF ELISA

The optimum concentration for SAS to be added to the dilution buffer was determined by using different concentrations ranging from 100 to 0.04%.

Because 100 μL of the sample was added to wells containing 50 μL of SAS-containing dilution buffer, the final concentration of SAS in the wells decreased to 1/3 of its original concentration.

Except for human serum, absorbance values of PBS-1% BSA, RPMI-10%FCS, and FCS samples did not significantly differ among all concentrations of SAS tested (Figure 1A).

When the effect of SAS was evaluated on PBS-1% BSA, RPMI-10%FCS, and FCS samples containing rhTNF-α at 330 pg/mL, no significant difference was observed up to 11% final concentrations of SAS. However, a dramatic increase was observed in the OD levels of human serum containing the same amount of rhTNF-α. SAS produced a concentration dependent

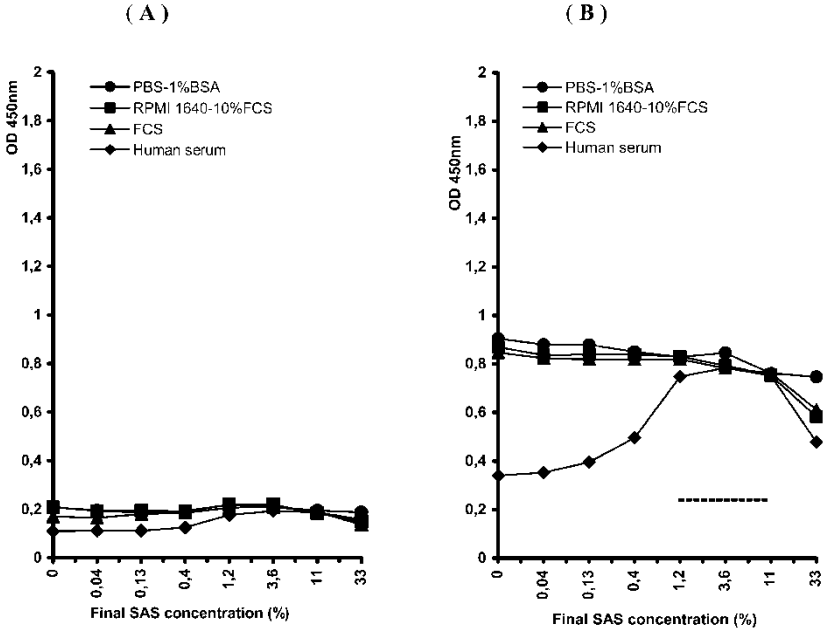

Figure 1. Effect of various concentrations of SAS on the OD levels of various samples assessed with TNF-alpha ELISA. 100 μL of different samples without (A) or with rhTNF-α at (B) 330 pg/mL was added to anti-hTNF-α mAb-coated wells containing 50 μL of dilution buffer with various concentrations of SAS. Further steps of the ELISA were performed as described in materials and methods section. Except for native human serum containing rhTNF-α, other samples did not demonstrate a prominent change in the OD levels by the addition of SAS up to the final concentration of 11%. However, dilution buffer containing SAS at the final concentrations between 1.2–11%, indicated by a horizontal dotted line, increased the OD levels of antigen containing human serum to a level nearly equal to that of other samples containing the same amount of antigen. Data are the means of triplicate wells of a representative experiment.

increase in the OD levels of human serum containing rhTNF-α at 330 pg/mL. More importantly, SAS at the final concentrations of 1.2–11% produced OD levels in human serum that were nearly equal to those obtained from other samples containing an equal amount of rhTNF-α (330 pg/mL) (Figure 1B). Hence, in all subsequent experiments, it was decided that dilution buffer containing SAS at 9% (which corresponded to 3% final concentration) seemed to be convenient to use as a component in the establishment and improvement of TNF ELISA for human serum. In addition, the effect of EDTA ranging from 0.09 to 60 mM was also analyzed. Similar effects were also obtained when EDTA was added to the sample diluent (data not shown).

Standard Curves of TNF ELISA Performed with Various Samples

Standard rhTNF-α was prepared at various concentrations, ranging from 1.3 to 1000 pg/mL in various samples. Figure 2 shows a comparison of the standard curves obtained from various samples with the presence of either dilution buffer without SAS (Figure 2A) or dilution buffer with 9% SAS (Figure 2B). Standard curves obtained for RPMI-10, FCS and heat-inactivated human serum, with either dilution buffer without SAS or dilution buffer with 9% SAS, were found to be very similar to each other.

A potential interference of complement proteins was evaluated by using heat-inactivated human serum and EDTA. Heat-inactivated human serum in dilution buffers, either with or without SAS, produced standard curves very similar, not just to each other, but also to those of RPMI-10 and FCS (Figure 2A and Figure 2B).

Performance Characteristics of TNF-α ELISA

Sensitivity

The standard antigen was diluted serially in human serum and the minimum detection limit, estimated by assaying the human serum four times, was less than 37 pg/mL (the mean + 2SD of the human serum without rhTNF was smaller than the mean-2SD of the human serum containing 37 pg/mL of rhTNF) and less than 12 pg/mL (the mean +2SD of the human serum without rhTNF which was smaller than the mean-2SD of the human serum containing 12 pg/mL of rhTNF) when dilution buffer without SAS and with 9% SAS were used, respectively (Table 1).

Reproducibility

Reproducibility values of TNF-α ELISA for human serum, performed using dilution buffer with 9% SAS, were measured as described here: The

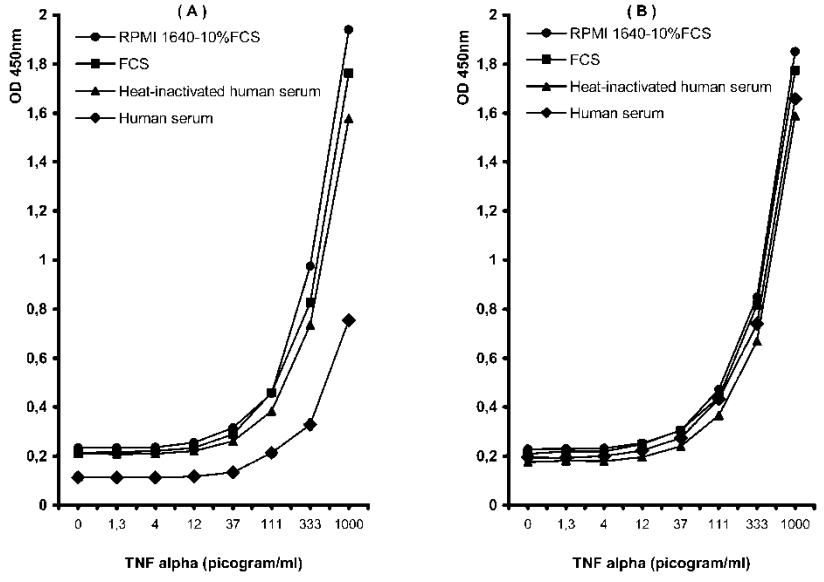

Figure 2. Standard curves of TNF alpha, constructed with various samples, using dilution buffer without (A) or with SAS (B) at 9%. Three-fold serial dilution of TNF was prepared in various samples and then measured with ELISA using 50 μL of dilution buffer with or without the present of SAS. The final concentration of SAS in the wells decreased to 3% when 100 μL of corresponding sample was added. Further steps of the ELISA were performed as described in the Experimental section. Except for native human serum, other samples, including heat-inactivated human serum, produced very similar standard curves in dilution buffer, either with or without the presence of 9% SAS. The low OD level standard curve of native human serum in dilution buffer without SAS was almost equalized to OD's of other samples by the addition of SAS into the dilution buffer at a concentration of 9%. Data are the mean of triplicate wells of a representative experiment.

Table 1. Effect of SAS on the analytical sensitivity of TNF-α ELISA

Dilution buffer	Without SAS		With 9% SAS	
Sample	RPMI-10[a]	Human serum	RPMI-10	Human serum
Minimum detection limit (pg/mL)[b]	<12	<37	<12	<12

[a]RPMI-1640 supplemented with 10% FCS.

[b]The Standard TNF-α prepared in various samples was diluted serially and the minimum detection limit was estimated as described in results section.

"intra-assay" coefficient of variance (CV), determined by assaying the human serum (containing rhTNF at 1000, 330, and 110 pg/mL) seven times, was found to be less than 6% (varied between 3.1%–5.8%). The "inter-assay" CV, determined by assaying human serum (containing rhTNF at 1000, 330, and 110 pg/mL) by four independent assays, was found to be less than 8% (varied between 4.6%–7.3%) (Data not depicted).

Recovery Rate

Table 2 summarizes the results of the recovery rates of hTNF-α ELISA for human serum performed using either dilution buffer without SAS or with 9% SAS. Recovery of TNF in human serum was improved by nearly 300% at 1000 pg/mL of antigen and even higher at lower concentrations of the standard that was added to the human serum.

Performance Characteristics of TNF-α ELISA with Human Serum and Plasma

Seven serum and 5 plasma (3 heparinized and 2 citrated) samples were assayed concomitantly with dilution buffers containing 9% SAS (final concentration of 3%) or only sample diluent consisting of PBS-1% BSA. A standard of rhTNF-α was run, simultaneously, for each assay system. Figures 3A and 3B depict the hTNF-α contents of the assayed human serum and human plasma, respectively. Both standard curves, run either with SAS or without

Table 2. Analytical recovery of TNF-α added to serum samples

Dilution buffer	TNF-α, pg/mL		% Recovery rate ((Y/X) × 100)
	Added (X)	Found[a,b] (Y)	
Without SAS	1,000	305 ± 42	30,5
	330	23.9 ± 11.86	7.25
	110	<1	<1
With 9% SAS	1,000	887.5 ± 57.9	87.75*
	330	287.95 ± 14.3	87*
	110	88.8 ± 9.2	80.7*

*Asterix denotes a significant difference ($p < 0.05$) when compared to its counterpart in dilution buffer without SAS.

[a]Measured using the standard curve constructed with heat-inactivated FCS containing TNFα between 12–1000 pg/mL.

[b]Mean ± SD value of four different serum samples (three replicates per specimen).

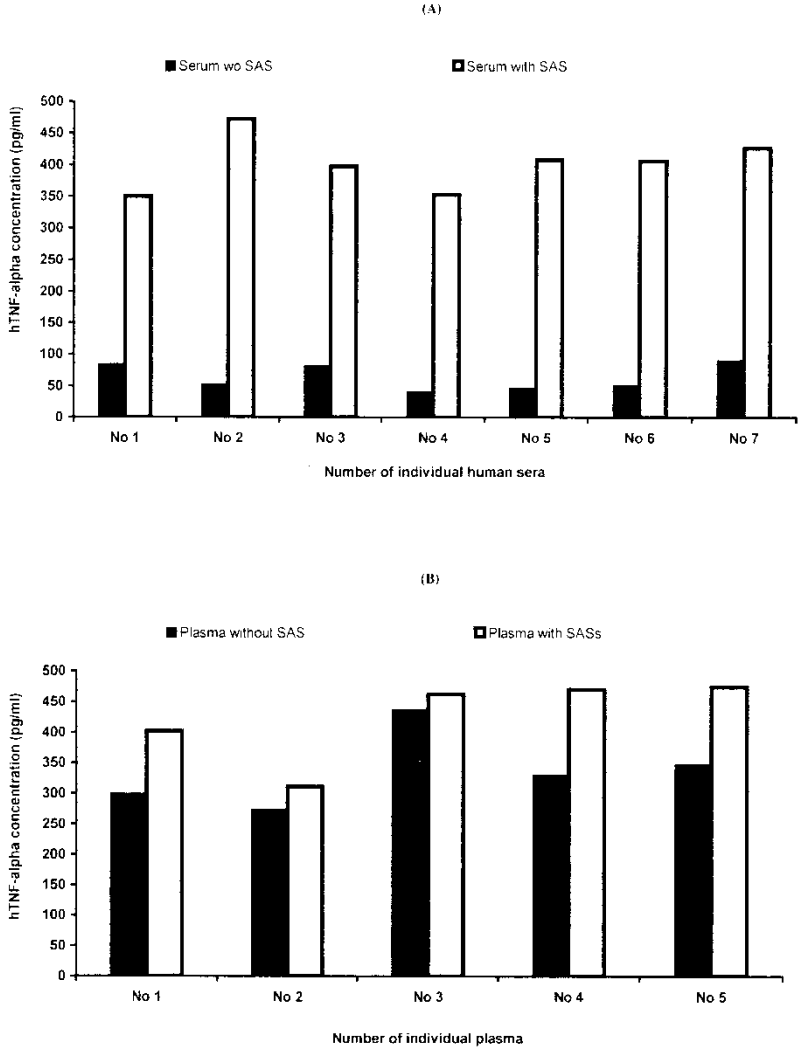

Figure 3. Bar graphs depicting 100 μL of serum (A) or plasma (B) samples each spiked with 500 pg/mL of rhTNF-α. Seven individual serum samples (Figure 3A) assayed with dilution buffers containing SAS (Figure 3A, empty bars) or without SAS (Figure 3A, filled bars) clearly show that the recovery rate is much higher and closer to the spiked concentration when SAS is introduced into the dilution buffer ($p < 0.001$). A similar effect of SAS was observed when human plasma, either heparinized (Figure 3B, numbers 1, 2, and 3) or citrated (Figure 3B, numbers 4 and 5), was assayed with dilution buffer containing SAS (Figure 3A, empty bars) or with only PBS-1% BSA (Figure 3A, filled bars) ($p = 0.012$). These results clearly show that SAS is effective in both serum and plasma for the recovery rates of rhTNF-α. Data are the means of quadriplicate wells of a representative experiment.

SAS containing dilution buffers, showed a highly comparable correlation. The hTNF-α content of the serum and the plasma were plotted and calculated against the corresponding standard rhTNF-α assays and multiplied by an inverse-dilution factor to yield the real contents.

In another assay system, performed simultaneously with the one mentioned above, SAS was also added to the conjugate. However, under these experimental conditions, performance characteristics of the assay did not further increase when various concentrations of SAS were additionally incorporated into biotinylated polyclonal anti human TNF-α mouse IgG conjugate (data not shown).

DISCUSSION

There are a number of problems associated with the development of an ELISA suitable for use in accurate measurement of cytokines in biological fluids. However, many of these same concerns can be raised for the use of bioassays for detection of molecules in biological fluids.[25] Despite the increasing popularity of immunoassays, there are many difficulties in achieving good analytical performance. Consequently, immunoassay results demonstrate poor interlaboratory comparability. To exclude these difficulties, several changes should be made; for example, preparation of common reference standards or means by which the "matrix effects" in immunoassay systems could be abolished.

An important interfering factor might be the complement proteins in serum samples. Although heat-inactivated foetal calf serum is regularly used as an "inert" additive for ELISA components and culture media, reconstitution of complement activity occurred on the addition of fractions of human serum.[26] Spuriously decreased values can be caused by the complement, which may interfere with antigen in binding to solid phase coated with antibody.[15,17]

During a study concerning heterophilic antibodies, we observed that SAS unexpectedly demonstrated an inhibitory effect on endogenous interference of native human serum; this observation was analyzed in detail and the results are presented in this report.

We have developed an in-house ELISA system for the measurement of hTNF-α in human serum, as well as in cell culture supernatants. It has a high analytical sensitivity limit and is highly comparable with commercially available ELISA systems in this regard. In addition, sensitivity of the assay for native human serum was found to increase at least three times when dilution buffer with 9% SAS was used (Table 1). The established ELISA system showed intra- or interassay CVs (3.1%–5.8% and 4.6%–7.3%, respectively), below 10%, which is highly suggestive of high reproducibility rates, an important feature and, in fact, a must for many immunoassays.

Although dilution buffer containing SAS higher than 0.4% increased the background OD level of human serum to some extent, this was not evaluated as a prominent change (Figure 1A). In addition, this result might provide an additional advantage because the background OD levels of human serum with dilution buffer containing SAS at 1.2–11% become very similar to that of other samples under the same experimental conditions (Figure 1A). Analytical recovery rates for TNF-α in human serum is clearly improved by the addition of 9% SAS (finally 3%) to the dilution buffer.

In the case of human serum with dilution buffer without SAS, a standard curve ($r^2 = 9977$, $p < 0.00001$) with low OD levels was obtained when compared to that of other samples. However, a dramatic correction in OD levels of the standard curve with human serum ($r^2 = 9964$, $p < 0.00001$) was obtained when dilution buffer with 9% SAS was used (compare Figure 2A and Figure 2B).

As expected, assays performed with EDTA-containing dilution buffer demonstrated the eliminated effects of complement proteins. Therefore, the results from the EDTA assays, in conjunction with the results drawn from the SAS assays, both showed that the endogenous interference of native human serum seemed to be related to the complement proteins.

Assaying individual human serum and plasma for their hTNF-α content revealed that addition of SAS increased the analytical sensitivity of the established ELISA system. Serum showing low or even undetectable hTNF-α concentrations when assayed with dilution buffer, without SAS, revealed relatively high concentrations when the assay was performed with SAS incorporated into the dilution buffer (Figure 3A). The same system established for human serum was used for heparinized or citrated human plasma. Plasma samples assayed with diluent, without SAS, showed relatively high hTNF-α concentrations in comparison to the serum samples assayed in parallel with diluent with SAS (Figure 3B). This may account for the fact that interfering factors present in freshly drawn serum highly inhibits the specific reactions expected to occur in our ELISA system. These reactions were reconstituted when SAS was used in the dilution buffer, which is suggestive of a role for the SAS for the inhibition of interfering serum factors, including complement proteins. The higher increase rates in citrated plasma, in comparison to heparinized plasma, may be attributed to the fact that heparin is a potentiating substance for factor H.

SAS has been used for long time as a protein precipitation agent. It has non-specific precipitative effects on protein groups, depending upon the concentration of SAS used. Figure 1A depicts the non-prominent effects of SAS on various samples without antigen. Except for human serum, no significant difference between the OD levels of various samples containing 330 pg/mL of rhTNF-α was observed up to 11% final concentration of SAS. However, a dramatic concentration dependent increase was observed in the OD levels of human serum containing the same amount (330 pg/mL) of rhTNF-α.

More importantly, dilution buffer containing SAS at 1.2–11% final concentrations produced OD levels in human serum with TNF at 330 pg/mL that was nearly equal to that obtained from other samples containing equal amount of antigen (Figure 1B). This suggests that the interference-abolishing effect of SAS for human serum is optimal at these concentrations. Standard curves obtained from human serum without SAS demonstrated a low-grade OD standard curve, whereas, a dramatic correction of standard curve with human serum was obtained when dilution buffer was used with final 3% SAS concentration. In addition, the defined amount of SAS increased the analytical sensitivity levels (Table 1) and recovery rates of antigen in human serum (Table 2). Under these experimental conditions, it seems to be rational and obvious that, for a precise measurement of TNF-α levels in human serum, defined amounts of SAS should be added into the dilution buffer.

Heating the serum sample abolishes the activity of complement proteins such as C1q[27] and is recommended for the reduction of interference for heat-stable analytes.[28] Optical density correction after heat-treatment, i.e., the so-called de-complementing of native human serum (Figure 2) showed that factors capable of causing interference seemed to be responsible for decreasing the performance characteristics of the ELISA system; they are converted to non-interfering substances after heating, either because of inactivation, or by precitipitation.

As a result, addition of SAS, at a defined concentration, to the dilution buffer eliminated the interference of native human serum, possibly the complement proteins, and created marked improvements in the ELISA used in this study. Although not presented here, similar results were also obtained when this method was applied to human interferon gamma ELISA. This method seemed to be an efficient alternative remedy to improve sensitivity and recovery rate of ELISA, at least for that susceptible to the inhibitory effect of complement proteins in native human serum.

ACKNOWLEDGMENT

This study was partially supported by a grant from TUBITAK (SBAG-AYD 427).

REFERENCES

1. Masseyeff, R.F. Standardization of immunoassays. Ann. Inst. Super. Sanita **1991**, *27* (3), 427–436.
2. Schrijver, R.S.; Kramps, J.A. Critical factors affecting the diagnostic reliability of enzyme-linked immunosorbent assay formats. Rev. Sci. Tech. **1998**, *17* (2), 550–61.

3. Rej, R.; Drake, P. The nature of calibrators in immunoassays: are they commutable with test samples? Must they be? Scand. J. Clin. Lab. Invest. Suppl. **1991**, *205*, 47–54.
4. Quinn, F.A.; Scopp, R.; Lach, A.; Drake, C.; Mo, M.; Albright, J.; Trimpe, K.A. Comparison of different sample matrices for evaluating functional sensitivity, imprecision and dilution linearity of the Abbott ARCHITECT i2000 TSH assay. Clin. Chem. Lab. Med. **2002**, *40* (7), 709–712.
5. Wood, W.G. "Matrix effects" in immunoassays. Scand. J. Clin. Lab. Invest. Suppl. **1991**, *205*, 105–112.
6. Andersson, G.; Ekre, H.P.; Alm, G.; Perlmann, P. Monoclonal antibody two-site ELISA for human IFN-gamma. Adaptation for determinations in human serum or plasma. J. Immunol. Meth. **1989**, *125* (1–2), 89–96.
7. Bjerner, J.; Nustad, K.; Norum, L.F.; Olsen, K.H.; Bormer, O.P. Immunometric assay interference: incidence and prevention. Clin. Chem. **2002**, *48* (4), 613–621.
8. Ismail, A.A.; Walker, P.L.; Cawood, M.L.; Barth, J.H. Interference in immunoassay is an underestimated problem. Ann. Clin. Biochem. **2002**, *39* (Pt4), 366–373.
9. Weber, T.H.; Kapyaho, K.I.; Tanner, P. Endogenous interference in immunoassays in clinical chemistry. A review. Scand. J. Clin. Lab. Invest. Suppl. **1990**, *201*, 77–82.
10. Kohse, K.P.; Wisser, H. Antibodies as a source on analytical errors. J. Clin. Chem. Clin. Biochem. **1990**, *28* (12), 881–892.
11. Madry, N.; Auerbach, B.; Schelp, C. Measures to overcome HAMA interferences in immunoassays. Anticancer Res. **1997**, *17* (4B), 2883–2886.
12. Kuroki, M.; Matsumoto, Y.; Arakawa, F.; Haruno, M.; Murakami, M.; Kuwahara, M.; Ozaki, H.; Senba, T.; Matsuoka, Y. Reducing interference from heterophilic antibodies in a two-site immunoassay for carcinoembryonic antigen (CEA) by using a human/mouse chimeric antibody to CEA as the tracer. J. Immunol. Meth. **1995**, *180* (1), 81–91.
13. Vaidya, H.C.; Beatty, B.G. Eliminating interference from heterophilic antibodies in a two-site immunoassay for creatine kinase MB by using F(ab′)2 conjugate and polyclonal mouse IgG. Clin. Chem. **1992**, *38* (9), 1737–1742.
14. Jones, S.L.; Cox, J.C.; Shepherd, J.M.; Rothel, J.S.; Wood, P.R.; Radford, A.J. Removal of false-positive reactions from plasma in an enzyme immunoassay for bovine interferon-gamma. J. Immunol. Meth. **1992**, *155* (2), 233–240.
15. Zimmermann-Nielsen, E.; Svehag, S.E.; Thorlacius-Ussing, O.; Baatrup, G. ELISA for evaluating the incorporation of plasma derived complement split-products C3b/iC3b into solid-phase immune complexes. J. Immunol. Meth. **2001**, *249* (1–2), 43–51.
16. van der Meide, P.H.; de Laibe, M.C.; Wubben, J.A.; Borman, A.H. Complement-mediated inactivation of interferon-gamma in ELISA systems. J. Immunoassay **1991**, *12* (1), 65–82.
17. Carlander, D.; Larsson, A. Avian antibodies can eliminate interference due to complement activation in ELISA. Ups. J. Med. Sci. **2001**, *106* (3), 189–195.
18. Larsson, A.; Wejaker, P.E.; Forsberg, P.O.; Lindahl, T. Chicken antibodies: a tool to avoid interference by complement activation in ELISA. J. Immunol. Meth. **1992**, *156* (1), 79–83.
19. Bormer, O.P. Interference of complement with the binding of carcinoembryonic antigen to solid-phase monoclonal antibodies. J. Immunol. Meth. **1989**, *121* (1), 85–93.

20. Boscato, L.M.; Stuart, M.C. Incidence and specificity of interference in two-site immunoassays. Clin. Chem. **1986**, *32* (8), 1491–1495.
21. Hudson, L.; Hay, F.C. Isolation and structure of immunoglubulins. In *Practical Immunology*; Blackwell Scientific Publications: Oxford, UK, 1976; 152–153.
22. Hebert, G.A.; Pelham, P.L.; Pittman, B. Determination of the Optimal Ammonium Sulfate Concentration for the Fractionation of Rabbit, Sheep, Horse, and Goat Antisera. Appl. Microbiol. **1973**, *25* (1), 26–36.
23. Mosedale, D.E.; Grainger, D.J. An antibody present in normal human serum inhibits the binding of cytokines to their receptors in an in vitro system. Biochem. J. **1999**, *343* (Pt1), 125–133.
24. Berg, K. Human interferon-gamma quantified via a sensitive one-site monoclonal antibody in a sandwich ELISA. A PEG modification for measurement in serum. APMIS **1994**, *102* (1), 13–22.
25. Kopp, W.; Reynolds, C.; Ruscetti, F. The immunoassay of cytokines and growth factors in biological fluids. Develo. Biol. Std. **1999**, *97*, 29–37.
26. Barkas, T.; Irvine, W.J. Bovine complement: formation of a lytic system between heat-inactivated foetal calf serum and fractions from normal human serum. J. Clin. Lab. Immunol. **1982**, *8* (2), 129–131.
27. Yoshida, H.; Imafuku, Y.; Nagai, T. Matrix effects in clinical immunoassays and the effect of preheating and cooling analytical samples. Clin. Chem. Lab. Med. **2004**, *42* (1), 51–56.
28. Selby, C. Interference in immunoassay. Ann. Clin. Biochem. **1999**, *36* (Pt 6), 704–721.

Received November 20, 2004
Accepted December 28, 2004
Manuscript 3149