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Optimization of analytical and pre-analytical variables associated with an ex vivo cytokine secretion assay

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Abstract

Purpose: Measurements of cytokine release in whole blood after ex vivo stimulation are useful in drug development. The components contributing to variation within such assays have not been clearly defined. Therefore, we characterized the sources of variability within an ex vivo stimulation assay for TNF-α release.
Method: Fresh whole blood or mononuclear cells from a cell preparation tube were added to silanized, screw-top tubes with a final concentration of 1 μg/mL lipopolysaccharide (LPS). Each tube was purged with 95% air/5%CO_2 and incubated 4 or 6 h at 37 °C in a metabolic water bath. Plasma TNF-α was next measured in supernatants by immunoassay. Total method variability was assessed in 10 normal donors each drawn in the morning and afternoon over 3 days. Four additional samples were pre-treated with dexamethasone to investigate inhibition of TNF-α release.
Results: Our analysis indicated precise temperature control, the timing and duration of stimulation, and the surface properties of the stimulation vessel most significantly influenced assay performance. A comparison of multiple anticoagulants indicated that careful consideration should be taken in selecting the optimal anticoagulant. The estimated total assay CV for all anticoagulants tested was less than 33.81%. The analytical variability (stimulation and measurement) was less than 25.88% CV. The one exception was mononuclear cells collected in sodium heparin. The total variability estimate incorporated day-to-day, diurnal, inter-donor, tube-to-tube and immunoassay variability. Using our optimized conditions, TNF-α release was inhibited by dexamethasone with a mean IC_{50} of 33.3 ± 4.6 nM.
Conclusions: We have described an optimal set of conditions for collection, storage and processing of an ex vivo cytokine stimulation assay. These conditions were selected for operational feasibility, minimal imprecision and elimination of potential confounding factors. The end result is a more robust method that can be applied to clinical drug development.

Keywords: Ex vivo stimulation; TNF-α; Lipopolysaccharide; Optimization

1. Introduction

Cytokines are key mediators of the immune response with central roles in the development, differentiation and regulation of immune cells. Changes in cytokine production are predictive of clinical outcome in selected disease states [1,2]. Therefore, quantification of cytokine production can provide a valuable biomarker tool for the identification of various diseases and for monitoring response to treatment. Accurate and reproducible methods for measuring cytokines, such as TNF-α, are critical for the application as clinical biomarkers for disease. Ex vivo cytokine secretion assays, where patient blood is exposed to an agonist and a response is measured, have proven to be a powerful tool for assessing cellular responses. Kirchner et al. first described ex vivo stimulation of cytokine release in whole blood in the early 1980s [3]. This technique has proven useful in identification of sepsis patients [4], determination of high

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and low cytokine producers [5], and provided pharmacological assessments of compounds in vivo and in vitro [6,7].

When monitoring cytokine levels with such an assay, an accurate estimate of assay imprecision and a comprehensive understanding of both the pre-analytical and analytical components contributing to assay variance is necessary to increase the likelihood of properly powering a study, such that statistically significant differences between treatment groups can be detected. A considerable body of evidence exists to suggest that the majority of factors contributing to total assay variability in the laboratory are pre-analytical [8]. Components contributing to pre-analytical variability in biomarker measurements can include such factors as patient gender, diet and biorhythms; sample collection procedures and preservatives; sample transport and storage conditions; specimen handling and processing techniques and assay specificity. Such factors ultimately influence the robustness of the assay and will negatively impact assay precision. Therefore, it is important to define pre-analytical variables which can be more tightly controlled, so their impact can be limited, and to fully understand those variables that cannot be controlled, so that their effects can be separated out from changes related to disease or therapeutic effect.

Van der Linden et al. conducted an evaluation of the overall laboratory error and individual variation within a whole blood assay for TNF-α cytokine release in response to LPS stimulation [5]. They determined that the total laboratory error from pre-analytical and analytical variables was at least three times smaller than the variation between individuals, the total assay variation was low enough to be able to distinguish between very low and very high cytokine producers. They also recommended heparin as the anticoagulant of choice for blood collection and that the stimulations be performed, uniformly for all samples, exactly 1 h after blood collection.

In the setting of clinical drug development, the majority of studies are conducted with samples collected at multiple sites, on multiple days, with multiple collections within a day. Overnight shipping of samples, prior to analysis, is often required. Therefore, it is not generally feasible to analyze all patient samples on the same day and it is necessary to maintain sample integrity over, at least, a 24-h period. In order to achieve the primary objective of therapeutic effect detection, we sought to clearly define the variance contribution of each component of the ex vivo cytokine stimulation assay to total assay variability and to optimize a procedure for sample collection and processing that minimizes variability in a clinically relevant setting, allows the detection of small, but relevant, changes between treatment groups while maintaining reasonable sample sizes.

2. Methods

2.1. Blood collection

Sample collection was performed within the guidelines for human research. All blood samples were obtained from normal volunteers through the research human blood donor program at Eli Lilly and Company. The following tubes were used for the experiments described: sodium heparin tubes (367874), sodium heparin cell preparation tubes (362753), sodium citrate cell preparation tubes (362761) or acid citrate dextrose solution B (364816) following the manufacturer's directions (Becton Dickinson, Franklin Lakes, NJ).

2.2. Reagents

Bacterial lipopolysaccharide preparation (LPS), isolated from *Escherichia coli* 026:B6 (D-1756) was purchased from Sigma Chemical (St. Louis, MO). Dulbecco's phoshate buffered saline pH 7.4 (DPBS, 14190-136) was purchased from Life Technologies, Gibco BRL (Gaithersburg, MD). Silanized tubes were purchased from Eagle Picher (Phoenix, AZ). TNF-α was measured using a bead-based sandwich immunoassay manufactured by (Linco Research, St. Charles, Missouri), and was analyzed on a Luminex 100 (Luminex Corporation, Austin, TX). Reagents for the TNF-α immunoassay were purchased from R&D Systems, (Minneapolis, MN).

2.3. Optimization experiments for ex vivo stimulation

Before running experiments aimed at optimizing collection and shipping conditions, a series of experiments was conducted to define the optimum set of conditions for factors that include heating devices, stimulation tube properties and time to stimulation. With each experiment 990 μL of whole blood, collected into sodium heparin tubes, was added to 10 μL of PBS with LPS (1 μg/mL final), the headspace above each sample was purged with a 95% air/5%CO_2, the tubes were sealed, and incubated for 4 h at 37 °C. In order to assess heating devices, samples from five donors were obtained at two time points 2 h apart. The samples were aliquotted, stimulated immediately, and incubated in either a Dubnoff metabolic water bath, a CO_2 cell culture incubator, or a dry heat, general-purpose laboratory incubator [9]. For the tube surface experiment, samples from four donors were obtained at two time points, 2 h apart. The samples were aliquotted into replicate tubes of both silanized and non-silanized borosilicate, stimulated immediately and incubated in a Dubnoff metabolic water bath at 37 °C. To examine delay to activation, two 8 mL samples from one donor were collected, held at 4 °C for defined amounts of time, aliquotted into silanized borosilicate tubes, and incubated in a Dubnoff metabolic water bath.

2.4. Optimized procedure

Nine hundred and ninety microliter whole blood was added to 10 μL LPS diluted in PBS to a final concentration of 1 μg/mL LPS in 13 mm × 100 mm silanized tubes. The headspace above each sample was purged with a 95% air/5%CO_2. Each tube was immediately sealed, then incubated in a Dubnoff metabolic water bath (Precision Scientific, Winchester, VA) at 37 °C for 4 h with shaking at 60 rpm. For mononuclear plasma from a CPT, the samples were incubated for 6 h instead of 4 h to enhance the TNF-α secretion. At the completion of the incubation, the samples were vortexed gently for 5 s and centrifuged at 850 × *g* for 5 min at 4°. The supernatant was removed and stored at −80 °C

for batch analysis by immunoassay. TNF-α was measured in the supernatant using Luminex technology and a human inflammatory cytokines multiplex assay kit [10].

2.5. Effect of storage temperature on ex vivo TNF-α production

A total of three heparin whole blood tubes and three heparin CPT were collected from three healthy volunteers. The samples were processed immediately, and an additional set of tubes from each volunteer were stored both ambient and at 4 °C overnight. The samples were stimulated and processed according to the optimized procedure.

2.6. Effect of anticoagulant on ex vivo TNF-α production

Sodium heparin and sodium citrate are the only anticoagulants available in CPT tubes, so they were evaluated in the ex vivo stimulation assay. Two heparin CPTs and two sodium citrate CPTs were collected from three healthy volunteers. Each anticoagulant was stored ambient and at 4 °C overnight. The samples were stimulated and processed according to the optimized procedure.

2.7. Estimate of precision

Blood was drawn into four anticoagulants from 10 normal donors, including samples from 6 males and 4 females, at 8:00 am and 4:00 pm on 3 consecutive days. After processing using the optimized collection and stimulation conditions, plasma was stored at −80 °C so that samples from each individual donor could be analyzed on the same plate. Inter-donor variation, diurnal variation, day-to-day variation and intra-donor variation were calculated.

2.8. Inhibition of TNF-α production by dexamethasone

The optimized assay was utilized to assess the pharmacodynamic effect of dexamethasone on TNF-α production. ACD whole blood was collected from four donors, aliquotted and incubated with 10 μL of 0–10 μM dexamethasone for 30 min in a Dubnoff at 37 °C. The headspace above each sample was purged with a 5% CO_2/air mixture, prior to incubation. Nine hundred and eighty microliter heparinized whole blood was added to 10 μL LPS diluted in PBS to a final concentration of 1 μg/mL LPS in 13 mm × 100 mm silanized tubes, using low binding tips. Samples were then processed according to the optimized procedure. All samples from a given donor were assayed on the same plate. IC_{50} values were calculated from concentration inhibition curves by non-linear regression analysis using Sigma Plot 8.0, Aspire (Leesburg, VA).

2.9. Statistical analysis

Data for assessing the total variability and the specific components of variability, such as inter-donor, inter-day, diurnal and intra-assay were analyzed within the framework of Random-Effects ANOVA model using JMP software of SAS Institute, Version 5.1, with the REML option for estimating the variance components. These analyses were performed after applying log (base 10) transformation to the TNF-α data in order to ensure approximate symmetry of the distribution.

3. Results

3.1. Impact of heating source on variability

A comparison of heating sources was conducted to determine the device that generated the most consistent values across two time points from the same donor. Ratios of TNF-α values were calculated (time point 2/ time point 1) for each donor and each device. A ratio of 1.0 would be expected for samples with absolute agreement. The results showed that incubation in the metabolic water bath, the CO_2 incubator, or the dry heat incubator generated mean ratios of 0.9, 0.85 and 5.8, respectively. Inter-individual ratios yielded coefficients of variation of 8.3% for the metabolic water bath, 35.4% for the CO_2 incubator and 30.5% for the dry heat incubator.

3.2. Comparison of silanized versus non-silanized tubes

A direct comparison was conducted between samples processed in silanized 13 mm × 100 mm screw cap tubes and non-silanized borosilicate 13 mm × 100 mm screw cap tubes. A 1.5-fold reduction in the coefficient of variation was obtained by using silanized tubes. The range of individual ratios for the silanized tubes was 0.81–1.08, and the mean ratio and standard deviation were 0.907 ± 0.124. The range for the non-silanized tubes was 0.71–1.12, and the mean and standard deviation were 0.942 ± 0.180.

3.3. Impact of sample processing time on TNF-α production

In early experiments, using sodium heparin blood collection tubes, we noted a decrease in TNF-α release in response to stimulation with increasing time between the sample draw and sample processing (data not shown). This result was similar to that seen by van der Linden et al. in their study [5]. To determine the impact of such delayed processing on TNF-α production, a normal donor sample was collected and held without processing for 0–28 h. Fig. 1a shows that when samples were processed after a 4 or 24-h delay, TNF-α release decreased 57 and 73%, respectively. To confirm the impact of a 24-h delay in processing, 10 separate donor samples were drawn, in duplicate, into sodium heparin tubes. One set of samples was processed immediately, and the other tube of each duplicate pair was held for 24 h at 4 °C prior to processing. A comparison of stimulated TNF-α release in these identical samples demonstrated a decrease of 64.7% in TNF-α release with a 24 h delay in processing ($12{,}377 \pm 386$ to 4376 ± 247 pg/mL) ($p < 0.001$), Fig. 1b. The values represent

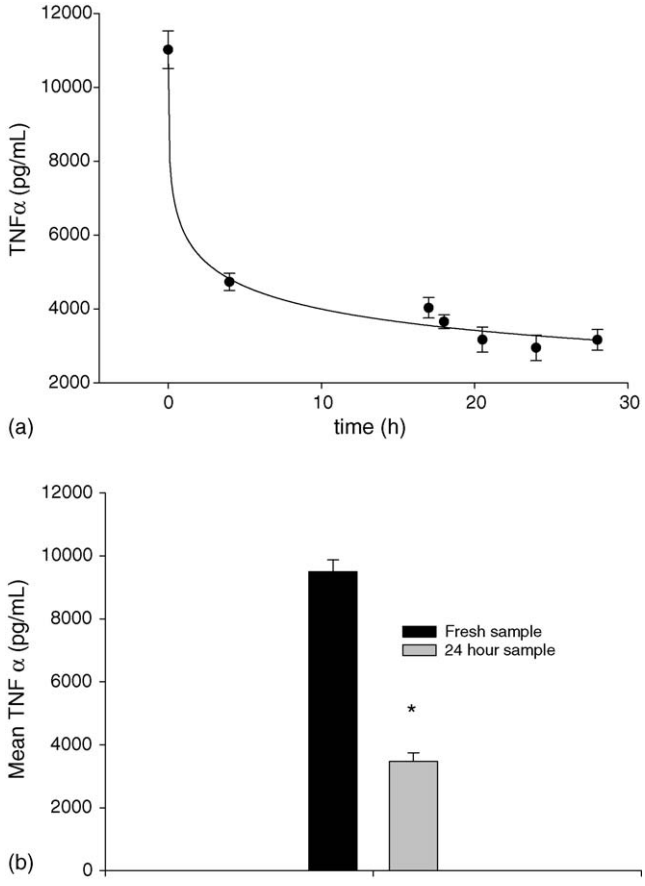

Fig. 1. (a and b) Sample processing delay. In (a), a normal donor had heparinized whole blood collected and processed at different times post-collection. The mean TNF-α concentration at each time point was determined from three tubes and plotted on the *Y*-axis. Error bars represent standard error of the mean (S.E.M.) for each time post-collection. In (b), 10 normal donors had samples processed both immediately and held for 24 h prior to processing according to the protocol. The mean TNF-α concentration for the population and the S.E.M. are plotted on the *Y*-axis. The dark bar represents immediate processing and the gray bar represents delayed processing. Asterisk (*) represents a statistically significant difference of $p < 0.001$ between immediate and 24 h delay.

the mean TNF-α concentration ± the standard error of the mean (S.E.M.).

3.4. Effect of storage temperature on ex vivo TNF-α production

Heparin whole blood and CPT plasma were stimulated immediately and also held ambient or at 4 °C for up to 48 h, to determine the effect of cold storage on TNF-α production. At 2 h, the mean TNF-α concentration of mononuclear cells and whole blood 4 °C stored samples demonstrated a 32% reduction (3078 ± 454 to 2103 ± 311 pg/mL). The ambient samples had only a 6% reduction (3109 ± 458 to 2902 ± 397 pg/mL) at the same time point. At 48 h the 4 °C stored samples had a 51% reduction (3078 ± 454 to 1517 ± 197 pg/mL), while the ambient stored samples had only an 8% reduction (3109 ± 458 to 2854 ± 290 pg/mL), Fig. 2. All values represent the mean TNF-α concentration ± S.E.M.

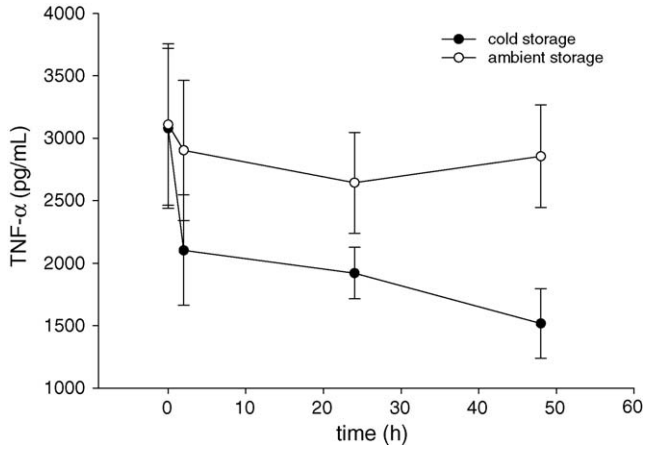

Fig. 2. Impact of cold storage on heparin collected samples. Replicate CPT and whole blood samples were drawn from three normal donors and processed immediately, 2, 24 and 48 h post collection. One sample was stored ambient on the laboratory bench and the corresponding sample was held at 4 °C. The cold stored mean TNF-α concentration for each time point (closed circles) and the ambient stored (open circles) and the S.E.M. were plotted on the *Y*-axis. The *X*-axis represents time in hours.

3.5. Effect of anticoagulant on ex vivo TNF-α production

Whole blood from sodium heparin tubes, stored at 4 °C demonstrated an increased loss in cytokine production over time as compared to sodium heparin tubes stored ambiently. Samples shipped at 4 °C can be more tightly temperature controlled than ambient samples (data not shown). Therefore, we questioned whether the use of a different anticoagulant might decrease the observed loss of cytokine production at 4 °C. To examine this, sodium heparin and sodium citrate CPT plasma were each collected and stored both ambient and at 4 °C overnight, prior to sample processing. Sodium heparin 4 °C stored samples had a 68% reduction in TNF-α production compared to the ambient stored samples (4888 ± 799 to 1550 ± 1312 pg/mL). The sodium citrate 4 °C samples, on the other hand, had 26% higher levels of TNF-α, compared to the sodium citrate ambient samples (2151 ± 635 to 2716 ± 601 pg/mL). The ambient sodium heparin was 227% higher than the corresponding sodium citrate sample, Fig. 3. All values represent the mean TNF-α concentration ± S.E.M.

3.6. Assessment of variation

It has been proposed that assays of the quasi-quantitative classification require assessment of assay precision and selectivity [11]. To address precision, components of variance analysis was determined from 10 normal donors, including samples from 6 males and 4 females, at 8:00 am and 4:00 pm on 3 consecutive days. Components of variance analysis showed that the greatest sources of variance for heparin whole blood, processed immediately, were inter-donor (20.72%) and diurnal (19.60%) factors. Total assay imprecision, composed of all sources of variation was 33.81% CV, and the total analytical coefficient of variation (composed of variance in the measurement and the tube to tube differences from the same sample collection) was 17.24% CV,

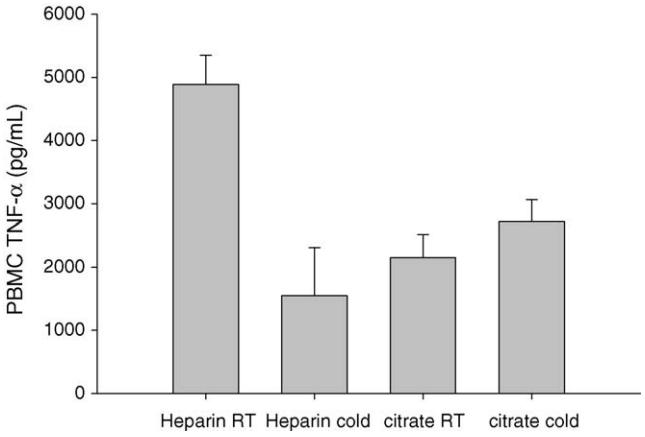

Fig. 3. Cold storage differences between anticoagulants. Three normal donors had both sodium heparin and sodium citrate CPT samples collected and stored ambient and at 4 °C overnight. The samples were processed according to an optimized protocol. The mean TNF-α concentration for each condition and the S.E.M. are plotted on the *Y*-axis. The *X*-axis represents each condition.

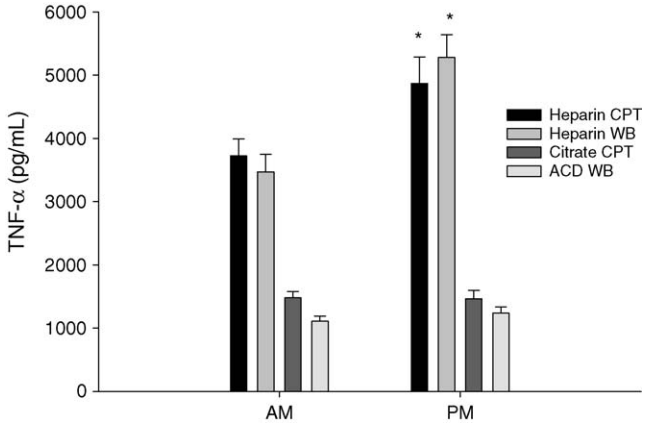

Fig. 4. Diurnal differences. Ten normal donors composed of six males and four females had samples collected into four different anticoagulants in the morning and the evening and processed according to the protocol. The mean TNF-α concentration for the heparin CPT (far left), heparin whole blood (second from the left), citrate CPT (third from the left) and ACD whole blood (far right) are plotted on the *Y*-axis. The error bars represent the S.E.M. The *X*-axis represents time of day AM or PM. The asterisks (*) represent statistically significant differences compared to the AM samples ($p < 0.05$).

Table 1. The sodium citrate-based anticoagulants have similar total assay variability with coefficients of variation of 30.42 and 33.37 for sodium citrate CPT and acid citrate dextrose (ACD), respectively, Table 1. The sodium citrate-based anticoagulant samples lack day-to-day variation and have negligible diurnal differences in this minimal assessment (Table 1), while both sodium heparin samples display a large component of diurnal variation that achieves statistical significance for (whole blood $p < 0.001$ and for CPT $p = 0.023$), Fig. 4.

3.7. Inhibition of TNF-α production by dexamethasone

To define assay selectivity, dexamethasone, a well-characterized inhibitor of cytokine release was added to demonstrate inhibition of TNF-α secretion following LPS stimulation [12]. IC_{50} values were extrapolated from inhibition curves, and ranged from 18 to 62 nM with a mean IC_{50} of 33.3 ± 4.6 nM, Fig. 5.

4. Discussion

Ex vivo stimulation assays are used throughout drug development for a variety of purposes, including defining compound efficacy in vivo [13]. Ex vivo, cell-based assays allow the assessment of numerous factors related to drug activity that are not accessible to most in vitro assays. Factors that are directly or indirectly measured in ex vivo stimulation assays, like the one described in this manuscript, include various cellular functions, amplification of target analyte and insights into the mechanism of action of the compound under study. In the case of ex vivo stimulation of cytokine release in peripheral blood, the assay platform is established, but the inherent variability of the assay has not been defined. Published methods are either not clinically applicable or necessitate relatively large sample sizes to achieve statistically rigorous results. We introduced several modifications to published methods that have improved the precision of the entire procedure.

Table 1
Components of variance

Components	CV (%)			
	Na heparin WB	Na heparin CPT	Na citrate CPT	ACD WB
Day	5.69	13.27	0.00	0.00
Diurnal	19.60	25.45	4.92	9.98
Subject	20.72	22.43	15.21	22.51
Residual[a]	17.24	31.53	25.88	22.52
Total	33.81	48.18	30.42	33.37

[a] Residual represents the variance associated with immunoassay and stimulation component.

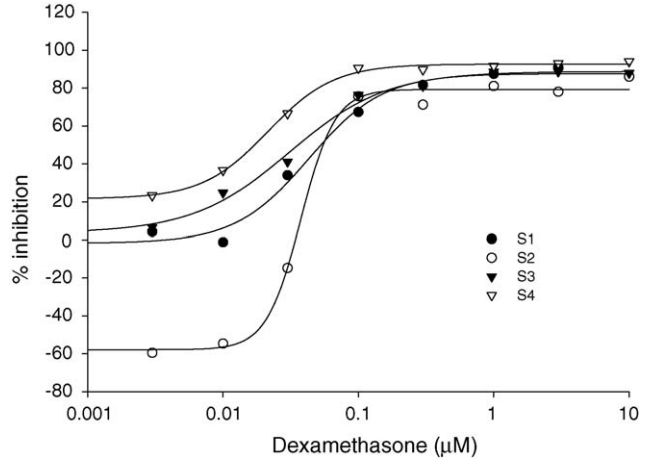

Fig. 5. Inhibition of TNF-α by dexamethasone. Four normal donors had ACD whole blood collected and aliquotted into 1 mL fractions and treated with different concentrations of dexamethasone. The TNF-α concentration was determined for each dose of dexamethasone and the percent of maximum inhibition was plotted on the *Y*-axis.

Our previous work with pancreatic beta cell stimulation demonstrated that tight control of cell stimulation conditions greatly reduced the variability of subsequent assays [14,15]. Parameters that have proven important in that context include: incubation temperature control, the surface properties of the vessel in which the stimulation is performed, temperature at which a sample is stored prior to stimulation and the type of anticoagulant used. Changes adopted here included changing the type of incubator, continuous gentle mixing, silanized surfaces and purging with a 5% CO_2/air mixture. Somewhat unexpectedly, the most significant improvement to the ex vivo stimulation protocol was the use of a Dubnoff metabolic water bath. A direct comparison of three heating sources demonstrated that the Dubnoff metabolic water bath had the lowest inter-donor coefficient of variation and a mean ratio of nearly 1.0 in repeat samples. The most plausible explanation for the improvement in precision with the Dubnoff metabolic water bath is the consistent transfer of heat and the improved thermal stability that water provides. In addition to heating source, tube surface properties are a significant source of imprecision. A comparison of non-silanized and silanized borosilicate tubes demonstrated improved reproducibility for silanized tubes in samples collected from the same donor 2 h apart. Silanized tubes provided an approximately 1.5-fold reduction in variability. This may be explained by a reduction in binding of monocytes and/or cytokines to the wall of the tube.

With an optimized assay in place, we defined the components of variability, addressed sample-handling concerns, confirmed assay selectivity and explored the application of the improved assay to clinical trial design. van der Linden et al. documented a considerable decrease in TNF-α concentration following a 2 h delay in processing of whole blood from sodium heparin collection tubes [5]. These results were corroborated by our laboratory in preliminary experiments and further confirmed in a comprehensive evaluation of 10 donors. Samples held for 24 h demonstrated a 67% decrease in TNF-α concentration. This loss of TNF-α production in heparin whole blood was a major concern for multi-site clinical trials, where samples are generally shipped overnight prior to processing. We established an alternative specimen collection and storage procedure. Storage condition experiments demonstrated a 32% reduction of TNF-α in 2 h compared to the immediately processed samples, and it only occurred in the refrigerated samples. An important consideration was to ensure that ambient shipping container could maintain a constant temperature. In data not shown, a temperature monitoring study revealed that ambient shipping recommendations were not adequate to maintain a constant temperature for 24 h during the winter months. A package shipped at 4 °C, however, was able to maintain constant temperature. Instead of engineering a device that could minimize temperature fluctuations, we chose to compare another anticoagulant, sodium citrate. In a direct comparison with sodium heparin, sodium citrate does not have the same reduction in TNF-α following refrigeration. In fact, the amount of TNF-α production was 26% higher when kept at 4 °C. The reason for the discrepancy in absolute production of TNF-α in heparin compared to citrate remains unsolved. Factors that could be contributing to the discrepancy and that warrant further investigation are the impact of cold on cell survival, extracellular calcium requirements for TNF-α production, and the anti-inflammatory properties of heparin. Since sodium citrate demonstrates a more consistent kinetic profile over time in the cold, it appears to be a more practical collection medium for shipping and storage of specimens collected at multiple clinical locations.

After making the analytical improvements and defining the pre-analytical variables, we defined the imprecision of the new method across multiple anticoagulants. The total assay imprecision for heparinzed whole blood, including donor-to-donor and diurnal variability, was 33.81% CV, and the analytical imprecision was 13.17% CV. In the heparin samples, day-to-day and diurnal variation existed that does not exist when collecting samples into sodium citrate anticoagulant. The citrate-based anticoagulants total assay precision estimates were very similar to heparin processed immediately (30.42% CV for CPT and 33.37% CV for ACD). Since the precision is comparable and the confounding variables have been reduced, citrate should be considered a more desirable anticoagulant for the clinical trial setting. The observed diurnal variation warrants further investigation.

We next examined the sensitivity of the assay to detect differences caused by a known anti-inflammatory agent. Dexamethasone treatment yielded a mean IC_{50} of 33 nM, a value that is consistent with other published reports [16]. These preliminary experiments indicate that the assay will detect inhibition in the therapeutic range of this reference anti-inflammatory agent.

Several issues relevant to application in clinical trials have been identified. Due to the loss of cytokine production with time between sample draw and stimulation in refrigerated heparin samples, ex vivo stimulation assays would require on-site stimulation or improved ambient shipping practices, however, the observation that citrate does not suffer from these same limitations make it a more appropriate anticoagulant. In addition, day-to-day variability and diurnal variation that exists in heparin collected samples would suggest that consideration must be given to time of collection and, most importantly, the inclusion of a placebo group. Given the limited sample size additional studies are needed further confirm this finding. Inclusion of a placebo group while optimal is not essential for citrate-based anticoagulants given the lack of day-to-day differences. Utilization of sodium citrate allows for dosing across an entire day or week without confounding variables.

In summary, we have defined assay improvements that reduce the total analytical variability of the whole blood assay. We have proposed a validation strategy for this assay type, and we have identified the ideal anticoagulant for multi-site clinical trials that eliminates many potential confounding variables.

References

[1] E. Hyjek, H.W. Lischner, T. Hyslop, J. Bartkowiak, M. Kubin, G. Trinchieri, D. Kozbor, J. Immunol. 155 (1995) 4060–4071.

[2] J.P. Mira, A. Cariou, F. Grall, C. Delclaux, M.R. Losser, F. Heshmati, C. Cheval, M. Monchi, J.L. Teboul, F. Riche, G. Leleu, L. Arbibe, A. Mignon, M. Delpech, J.F. Dhainaut, J. Am. Med, Assoc. 282 (1999) 561–568.

[3] H. Kirchner, Ch. Kleinicke, W. Digel, J. Immunol. Methods 48 (1982) 213–219.
[4] W. Heagy, C. Hansen, K. Nieman, M. Cohen, C. Richardson, J.L. Rodriguez, M.A. West, Shock 14 (2000) 271–276.
[5] M.W. van der Linden, T.W.J. Huizinga, D. Stoeken, A. Sturk, R.G.J. Westendorp, J. Immunol. Methods 218 (1998) 63–71.
[6] S.A. Wadsworth, et al., J. Pharmacol. Exp. Ther. 291 (1999) 680–687.
[7] M.H. Magee, R.A. Blum, C.D. Lates, W.J. Jusko, Br. J. Clin. Pharmacol. 53 (2002) 474–484.
[8] P. Bonini, M. Plebani, F. Ceriotti, F. Rubboli, Clin. Chem. 48 (5) (2002) 691–698.
[9] S. Chow, H. Patel, D.W. Hedley, Cytometry 46 (2001) 72–78.
[10] C.A. Ray, R.R. Bowsher, W.C. Smith, V. Devanarayan, M.B. Willey, J.T. Brandt, R.A. Dean, J. Pharm. Biomed. Anal. 36 (2005) 1037–1044.
[11] J.W. Lee, G.D. Nordbloom, W.E. Smith, R.R. Bowsher, in: JC. Bloom, RA. Dean (Eds.), Biomarkers in Clinical Drug Development, Marcel Dekker, NY, 2002, pp. 119–149.
[12] N. Martinet, P. Vaillant, T. Charles, J. Lambert, Y. Martinet, Eur. Respir. J. 5 (1992) 67–72.
[13] D.A. Parasrampuria, P. de Boer, D. Desai-Krieger, A.T. Chow, C.R. Jones, J. Clin. Pharmacol. 43 (4) (2003) 406–413.
[14] R.J. Konrad, K. Liu, J.E. Kudlow, Arch. Biochem. Biophys. 381 (2000) 92–98.
[15] R.J. Konrad, I. Mikolaenko, JF. Tolar, K. Liu, JE. Kudlow, Biochem. J. 356 (2001) 31–41.
[16] D. Franchimont, E. Louis, W. Dewe, H. Martens, Y. Vrindts-Gevaert, D. De Groote, J. Belaiche, V. Geenen, Regul. Pept. 73 (1998) 59–65.