Whole Blood-Based Multiplex Immunoassays for the Evaluation of Human Biomarker Responses to Emerging Viruses in Resource-Limited Regions

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Abstract

Many emerging viruses such as Ebola and Lassa occur in resource-limited areas of the world. The advent of multiplex immunoassays has facilitated the study of biomarkers of disease since only small amounts of clinical material are required; however, such assays are designed and validated for only plasma or serum. This is a significant impediment when studying infectious diseases in the context of an outbreak in a developing nation. Plasma or serum can be difficult to obtain in the field due to the need for additional processing of infectious materials. Evaluation of multiplex immunoassays using frozen and thawed human whole blood (WB) would permit additional analysis using a more readily available human clinical sample. In this study, frozen and thawed human WB was directly compared with frozen and thawed plasma from normal healthy donors in a series of multiplexed immunoassays for 59 different biomarkers. We demonstrate that most important biomarkers can be evaluated using thawed WB, which will facilitate the study of human cytokine and other biomarker responses to viruses emerging in resource-limited regions.

Keywords: multiplex immunoassay, whole blood, plasma, biomarker, cytokine, emerging virus

Introduction

THE STUDY OF HUMAN biomarkers as they relate to disease manifestations or patient outcomes in the context of infection with the hemorrhagic fever viruses is an important and expanding area of research. However, small volumes of clinical material and the infectious nature of the pathogens have limited studies to date even in resource-rich settings (4). The infectious nature of the pathogen leads to an effort to minimize phlebotomy due to the risk of secondary exposure, and subsequently the need for many different tests to be performed on the same blood sample. This has been overcome, in part, by the commercial availability of different multiplex immunoassays that permit analysis using only very small volumes of clinical sample (2,8). Progress has been made in recent years using these types of assays to look at cytokines and chemokines as well as other biomarkers of human disease pathophysiology, including coagulopathy and endothelial dysfunction (5–7). However, the requirement to have serum or plasma to perform these multiplex immunoassays continues to hamper scientific progress in this area. This is a direct result of the difficulty in processing infectious blood in the field; it is often only possible

to cryopreserve whole blood (WB) during an outbreak response. Although WB has been used in many different diagnostic assays in the past, including nucleic acid-based and serologic assays, a search of the literature did not reveal any studies in which WB is directly used in multiplex immune assays. In all available literature, WB is separated to produce plasma or serum before analysis. Even in studies that have extensively examined the stability of various cytokines based upon the anticoagulant, storage time, and temperature, the plasma has always been separated from the blood cells before cryopreservation (3). This does not adequately represent how samples are handled in the field. In a field situation, a blood sample is obtained from a clinical site, usually in an EDTA, citrate, or a heparin tube and transported to the laboratory. It is worth noting that for PCR-based diagnostic testing, heparin tubes should not be used because it can interfere with the assay (9). In addition, plasma obtained from heparinized tubes exhibited greater concentration variability in multiplex immunoassays than that observed with EDTA or citrate (1). Two other factors that are especially relevant to field studies include the duration of time from collection to arrival in the laboratory and the environmental conditions under which the sample is

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stored during transport. This can be affected by many variables, such as distance, mode of transport, and frequency of couriering. Once received in the laboratory, the sample is immediately processed for diagnostic analysis and then the remaining blood is transferred to a storage tube and stored at $-20^{\circ}\text{C}/-80^{\circ}\text{C}$ or in liquid nitrogen if available. Centrifugation in the field setting introduces too much risk for it to be routinely performed, so it is not typical to obtain plasma or serum. Unfortunately, all commercially available multiplex immunoassays are validated only for plasma or serum and it is unknown how a freeze-thaw cycle on WB would affect the levels of various biomarkers in the sample. Therefore, in this study, we sought to assess whether WB that had been thawed after freezing would be an acceptable alternative matrix for use in these assays—thus utilizing a system in which the processing was similar to a field setting.

Methods

WB was obtained in acid citrate dextrose tubes from eight healthy donors as part of an approved human subjects research protocol for normal healthy donor phlebotomy (CDC IRB 1572). Blood from each donor was split into half: one half was aliquoted and frozen at -80°C . The other half was centrifuged at $\sim 2,000$ rpm for 5 min at room temperature in a table top microfuge to separate plasma from red blood cells (RBCs), and the plasma was collected, aliquoted, and frozen at -80°C . These frozen paired WB and plasma samples were thawed, centrifuged at max speed in a table top microfuge for 10 min to remove any membrane particles, and the supernatants were used in the assays. It is important to note that because there was not an appreciable pellet after this process, some membrane particles could still be contained in the WB sample, although they would be very small. In addition, these preparation conditions represent an ideal scenario in which blood is collected, transported, and processed within a few hours. This may not always be possible in a field setting. Supernatants from both WB and plasma preparations were then run through a series of commercially available multiplex immune assays. Fifty-nine different biomarkers were assessed in 10 assays. Each assay was performed according to the manufacturers' instructions. The largest of these was a 30-plex assay for G-CSF, GM-CSF, IFN- α , IFN- γ , IL-1 β , IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, TNF- α , Eotaxin, IP-10, MCP-1, MIG, MIP-1 α , MIP-1 β , RANTES, EGF, FGF-basic, HGF, and VEGF that was performed using an overnight incubation (Invitrogen). Five assays also incubated overnight included single-plex assays for Ferritin, ADAMTS13, and CFH, a two-plex assay for tissue factor and thrombomodulin, and a four-plex assay for vWF, PF4, CRP, and fibrinogen (Millipore). An IFN- β single-plex assay, an 11-plex assay for E-selectin, fractalkine, granzyme B, GRO- α , IL-29, L-selectin, MCP-2, MCP-3, sCD40L, TNF-R2, and tPA, and a 6-plex assay for D-dimer, PAI-1, PECAM, P-selectin, sFas-ligand, and TNF-R1 were performed using a 2-h incubation (Affymetrix). A two-plex assay for intracellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) was run with an overnight incubation (Affymetrix); however, due to the inability to consistently recover sufficient bead counts after multiple attempts, ICAM was excluded from further anal-

ysis. Most assay results were reported in pg/mL or ng/mL; D-dimer, CRP, and fibrinogen were converted to their commonly used units of $\mu\text{g/mL}$, mg/L, and mg/dL, respectively. The manufacturer-provided standard curves were evaluated by five parameter logistic regression. The r^2 values were >0.95 for all analytes with the following exceptions: sCD40L $r^2=0.90$, E-selectin $r^2=0.69$, TNFR1 $r^2=0.85$, VCAM $r^2=0.72$, and IFN-beta $r^2=0.88$. Only kits manufactured by Millipore have low and high concentration quality controls included, and in all cases, the measured values were within the expected ranges. Two outliers were excluded from analysis for IL-29, MCP-2, and tPA.

Results

Biomarker values were within the measurable range for the majority of the samples ($>60\%$ for both WB and plasma) for 35 of the 59 biomarkers evaluated. The 24 biomarkers that were below the limit of detection for most samples included IL-1 β , G-CSF, IL-10, IL-13, IL-6, IL-17, GM-CSF, IL-5, VEGF, TNF- α , IL-2, IL-7, IP-10, MIG, IL-4, IL-8, Fractalkine, MCP-3, Granzyme B, sCD40L, sFas-ligand, tissue factor, thrombomodulin, and IFN- β . This finding is consistent with earlier work conducted by our group using plasma from normal healthy humans with the exception of GM-CSF, IL-2, TNF- α , and IL-5 that were previously routinely detected above the assay limit of detection, although a different manufacturer's assay was used for these analytes in those previous studies. In this study, IFN- β was performed in a 2 h assay compared with previous work in which the assay was performed as an overnight incubation, which increases the sensitivity of the assay; this explains why IFN- β was below the limit of detection in our samples. Thrombomodulin was below the limit of detection in the samples used in this study but had been detectable in prior studies, possibly due to its membrane association and that these samples were centrifuged before analysis, which was not performed in previous work.

The differences that were observed between WB and plasma measurements were statistically significant for 21 of the 35 detectable biomarkers by the Student's t -test; this includes interferons, cytokines, chemokines, other inflammatory markers, markers of coagulopathy, markers of endothelial function, and growth factors (Table 1). All raw data are shown for each paired set of WB and plasma. The most notable differences were seen for IL-1RA that was a log higher in WB, and MCP-2 that was lower in WB than in plasma. These two analytes, along with IL-2R, which exhibited no statistical difference between WB and plasma, are depicted in Figure 1.

Discussion

The process of freezing WB results in lysis of the RBCs and white blood cells (WBCs) that are contained within the sample. Any biomarker contained within the cell would be released into the supernatant during this process and could affect the results. This is most important when assaying for biomarkers that might be produced by WBCs, such as cytokines and chemokines, or when assaying for iron storage products that might be higher in RBCs. This seems to be the case for IL-1RA, but was not as obvious with any of the other measurable immune markers, despite the fact that some differences were statistically significant. An additional

TABLE 1. COMPARISON BETWEEN PLASMA AND WHOLE BLOOD IN MULTIPLEX ASSAYS

Analyte	Assay LOD	Units	Plasma range	Plasma mean	WB range	WB mean	p-Value
ADAMTS13*	122	ng/mL	422–762	555	220–872	391	0.01
CFH*	4,883	ng/mL	176,801–276,624	225,882	80,983–211,069	161,698	0.0002
CRP	0.5	mg/L	0.5–28	7	0.5–17	5	0.08
D-dimer*	0.003	µg/mL	0.2–1.3	0.7	0.3–3.1	1.7	0.004
E-selectin	250	pg/mL	8,901–67,177	43,226	7,138–61,337	40,112	0.08
EGF*	18	pg/mL	18–288	71	33–354	90	0.03
Eotaxin*	10	pg/mL	24–59	40	27–140	68	0.03
Ferritin*	2	ng/mL	26–117	61	38–163	87	0.03
FGF-basic	12	pg/mL	17–851	135	23–967	150	0.3
Fibrinogen*	0.1	mg/dL	143–241	191	60–143	109	<0.0001
GRO α *	2	pg/mL	9–15	12	9–14	11	0.03
HGF	41	pg/mL	232–960	414	214–1,290	426	0.8
IFN- α	22	pg/mL	22–70	31	24–86	37	0.09
IFN- γ *	14	pg/mL	15–48	35	36–55	47	0.0003
IL-12 (p40/p70)*	21	pg/mL	54–120	90	32–91	61	0.0002
IL-15	118	pg/mL	118–2,568	555	118–3,666	654	0.5
IL-1RA*	110	pg/mL	110–217	168	2,154–6,640	4,196	0.0002
IL-29 (IFN- λ)	57	pg/mL	111–144	125	100–122	111	0.1
IL-2R	64	pg/mL	178–382	255	168–323	240	0.3
L-selectin*	38	pg/mL	14,507–33,549	24,671	19,854–34,025	27,570	0.003
MCP-1*	76	pg/mL	138–443	243	138–543	383	0.01
MCP-2*	0.5	pg/mL	3–9	6	2–4	3	0.002
MIP-1 α	50	pg/mL	50–175	77	50–248	84	0.3
MIP-1 β	22	pg/mL	35–2,494	392	39–2,716	407	0.6
P-selectin*	1,233	pg/mL	9,510–78,642	24,796	15,273–68,604	45,232	0.02
PAI-1*	19	pg/mL	2,176–4,354	3,332	3,513–7,989	5,923	0.0006
PECAM-1*	407	pg/mL	12,601–28,591	22,029	13,393–22,612	18,124	0.002
PF4*	366	ng/mL	366–4,234	1,635	3,078–8,000	5,612	0.0001
RANTES	53	pg/mL	460–951	687	472–1,784	933	0.07
TNF-R1*	170	pg/mL	4,074–6,959	5,555	1,083–5,098	2,694	0.0006
TNF-R2*	1	pg/mL	93–247	166	85–196	132	0.04
tPA	39	pg/mL	244–572	389	230–405	347	0.3
VCAM*	1,890	pg/mL	73,511–308,212	164,569	43,797–117,551	78,364	0.01
vWF	2,436	ng/mL	3,981–11,923	6,748	3,638–9,791	6,525	0.8

Starred (*) analytes indicate a p -value <0.05 as determined by the paired Student's t -test.

LOD, limit of detection; IFN, interferon; IL, interleukin; GRO, growth-regulated protein; MIP, macrophage inflammatory protein; MCP, macrophage chemoattractant protein; RANTES, regulated upon activation, normal T cell expressed and secreted; CFH, complement factor H; CRP, c-reactive protein; TNF, tumor necrosis factor; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; PAI, plasminogen activator inhibitor; PF, platelet factor; tPA, tissue plasminogen activator; vWF, vonWillebrand factor; PE-CAM, platelet and endothelial cell adhesion molecule; VCAM, vascular cell adhesion molecule; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; ICAM, intracellular adhesion molecule, did have detectable levels in all samples but was excluded from this analysis due to insufficient bead counts.

FIG. 1. Representative graphic examples of whole blood versus plasma concentrations of several biomarkers. Examples of the three different outcomes are shown; IL-1RA was significantly higher in whole blood, MCP-2 was significantly higher in plasma, and IL-2R had no significant differences between measured levels in either matrix. Dotted line represents the limit of detection of the assay. p -Value obtained using the Student's t -test is noted on each graph. MCP, macrophage chemoattractant protein.

consideration is that plasma constitutes ~55% of blood volume, whereas the cells are ~45%. Therefore, measurements made in WB might be “diluted” relative to the plasma since the volume used in each assay was identical.

Each of these assays uses two antibody pairs to detect a given analyte. One antibody is coupled to a unique magnetic bead and captures the analyte that is present in the sample. The second antibody is coupled to biotin and the complex is detected with a streptavidin molecule linked to the fluorophore phycoerythrin. When a particular manufacturer develops a commercial assay, they may not use the same two antibodies for capture and detection as another manufacturer, and this could influence the sensitivity of the assay. This has been observed in our studies and underscores the importance of establishing a series of normal values for any particular sample type in a given assay to permit appropriate interpretation of the results. Since most analyses are conducted in patients with different diseases, it is always helpful to have a cohort of normal healthy subjects for comparison.

The most important finding in this study was that frozen and thawed human WB is an acceptable matrix for multiplex immunoassays. The fact that there are statistically significant differences between the concentration values obtained in WB versus plasma underscores the importance of including a cohort of normal healthy subjects using the same sample matrix for comparison. The normal values obtained from frozen and thawed WB samples in these assays only apply to these specific manufacturers’ assays performed with the same incubation duration, and should not be extrapolated to other manufacturers’ assays or to different incubation times. These assays were not performed on fresh WB. Fresh WB contains cells with intact membranes that have the ability to respond to stimulation and the potential to release additional biomarkers that could complicate interpretation of the data.

For investigators who study emerging viruses such as the hemorrhagic fever viruses or other contagious pathogens that occur in spontaneous outbreaks in the developing world, this study will prove quite useful. It solves one issue related to the limited resources that are found in the developing world outbreak scenario—no need for additional manipulations or centrifugation of the samples, thus making the process of storing samples safer and easier. Freezing of the sample will still be necessary for integrity of the assays and for the intentional lysis of the cells contained within a WB preparation. This protocol does not obviate the need to transfer the sample to a laboratory with appropriate containment and technical resources, but it makes collection of the sample in the field much more practical.

Going forward it will be important to conduct these same assessments in stimulated samples to permit for analysis of additional biomarkers that were not detectable in normal healthy humans. In addition, greater samples sizes and evaluation of the effects of different coagulants on the measured concentrations are also needed.

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Author Disclosure Statement

All authors declare that no competing financial interests exist.

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