

TRANSLATIONAL PHYSIOLOGY |

Whole blood and leukocyte RNA isolation for gene expression analyses

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Submitted 2 February 2004; accepted in final form 10 May 2004

Feezor, Robert J., Henry V. Baker, Michael Mindrinos, Doug Hayden, Cynthia L. Tannahill, Bernard H. Brownstein, Adrian Fay, Sandra MacMillan, Jason Laramie, Wenzhong Xiao, Lyle L. Moldawer, J. Perren Cobb, Krzysztof Laudanski, Carol L. Miller-Graziano, Ronald V. Maier, David Schoenfeld, Ronald W. Davis, Ronald G. Tompkins; and the Inflammation and Host Response to Injury, Large-Scale Collaborative Research Program. Whole blood and leukocyte RNA isolation for gene expression analyses. *Physiol Genomics* 19: 247–254, 2004; doi:10.1152/physiolgenomics.00020.2004.—The analysis of gene expression data in clinical medicine has been plagued by the lack of a critical evaluation of accepted methodologies for the collection, processing, and labeling of RNA. In the present report, the reliability of two commonly used techniques to isolate RNA from whole blood or its leukocyte compartment was compared by examining their reproducibility, variance, and signal-to-noise ratios. Whole blood was obtained from healthy subjects and was either untreated or stimulated *ex vivo* with *Staphylococcus* enterotoxin B (SEB). Blood samples were also obtained from trauma patients but were not stimulated with SEB *ex vivo*. Total RNA was isolated from whole blood with the PAXgene proprietary blood collection system or from isolated leukocytes. Biotin-labeled cRNA was hybridized to Affymetrix GeneChips. The Pearson correlation coefficient for gene expression measurements in replicates from healthy subjects with both techniques was excellent, exceeding 0.985. Unsupervised analyses, including hierarchical cluster analysis, however, revealed that the RNA isolation method resulted in greater differences in gene expression than stimulation with SEB or among different trauma patients. The intraclass correlation, a measure of signal-to-noise ratio, of the difference between SEB-stimulated and unstimulated blood from healthy subjects was significantly higher in leukocyte-derived samples than in whole blood: 0.75 vs. 0.46 ($P = 0.002$). At the $P < 0.001$ level of significance, twice as many probe sets discriminated between SEB-stimulated and unstimulated blood with leukocyte isolation than with PAXgene. The findings suggest that the method of RNA isolation from whole blood is a critical variable in the design of clinical studies using microarray analyses.

microarray; buffy coat; PAXgene; inflammation; trauma

DESPITE SIGNIFICANT PROGRESS in medical science and basic biological research, our ability to understand the pathological basis of human disease remains limited. Understanding the host response to severe trauma and injury, for example, has been hindered by the lack of integrated technologies that can reveal its complexity and diversity. And yet, the clinical problem of trauma and injury is enormous. Over 6,000,000 individuals are hospitalized annually because of trauma, injury, and burns (1). Although most of these individuals survive, a significant number go on to develop late complications, including sepsis and multisystem organ failure.

One approach to broaden our understanding of the pathological basis for complex human diseases is to apply novel high-throughput genomic technologies. At present, these technologies, including large-scale expression profiling, have been applied to differentiate histologically similar tumors, monitor disease progress, and individualize treatment regimens in a variety of neoplastic diseases (6, 7, 11, 13, 17). But the absence of a coordinated strategy to integrate new technologies into other aspects of medical science, and particularly into an analysis of the host response to injury or disease, has limited our ability to realize the full potential of these new technologies.

The National Institute of General Medical Sciences (NIGMS), in a pioneering effort to facilitate a better understanding of the host response to severe trauma and burn injury, has supported a Large-Scale Collaborative Research Program (“Glue Grant,” see <http://www.nigms.nih.gov/funding/gluegrants.html>) titled “Inflammation and the Host Response to Injury” (see <http://www.gluegrant.org/>). One of its goals is to use gene expression profiles obtained from circulating blood leukocyte populations to predict which trauma and burn patients will go on to develop sepsis and multisystem organ failure. A second goal is to further advance the basic understanding of the innate immune and inflammatory responses to trauma and burns by dissecting leukocyte gene expression patterns in blood to

Article published online before print. See web site for date of publication (<http://physiolgenomics.physiology.org>).

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define the common and injury-specific host responses to systemic inflammation.

In the initial phase of the program, and in the absence of widely accepted protocols and methodologies directed at the collection, processing, labeling, and hybridization of nucleic acid samples obtained from clinical materials, a programmatic decision was made to establish standard operating procedures for the isolation of RNA from whole blood and blood leukocytes. The development of these robust protocols and the clear and thorough elucidation of their advantages and limitations should provide the requisite foundation for this and other future efforts in translational medicine.

To optimize and validate the collection and isolation of RNA from whole blood or whole blood leukocytes, we assessed the reliability, signal-to-noise ratios, and variance associated with gene expression profiles obtained from healthy subjects and from trauma patients using a traditional leukocyte isolation procedure and a whole blood RNA collection system. To approximate changes in gene expression that are likely encountered in vivo by patients with microbial infection following severe injury, blood samples from healthy subjects were incubated ex vivo with *Staphylococcus* enterotoxin B prior to stabilization and/or leukocyte RNA isolation.

Whole blood RNA collection systems are an attractive approach in the clinical setting because of their ability to stabilize nucleic acids immediately upon collection without the need to extract the blood leukocyte compartment. One such system, PAXgene (PreAnalytiX, Hombrechtikon, Switzerland), has been shown to prevent RNA degradation and to restrict ex vivo gene expression in anticoagulated whole blood when stored at room temperature or 4°C for periods up to 5 and 90 days, respectively (10). PAXgene offers a number of technical advantages in clinical studies including its ease of use, reduced operator time, and reduced risk of exposure of blood products to laboratory personnel. PAXgene has been used to assess whole blood mRNA levels with either RT-PCR or microarray analyses (9, 10, 12, 14). These studies have shown the technique to be highly effective at stabilizing mRNA in whole blood for long periods of time with little effect on the subsequent RT-PCR or microarray analyses. However, little is known regarding the reproducibility, variance, and signal-to-noise ratios associated with these whole blood RNA collection methodologies. Furthermore, it is unclear whether they are comparable to methods that first isolate leukocyte enriched cell populations. The present studies were therefore undertaken to conduct a systematic comparison between a whole blood and leukocyte RNA collection system for their ability to detect changes in gene expression. At present, there is no consensus regarding the optimal technique to isolate RNA from blood cell populations. Such an evaluation would provide the basis for subsequent studies to be conducted in multicenter studies of critically ill patients or other hospitalized patient populations.

MATERIALS AND METHODS

Blood collection and study design. Permission to obtain venous blood from healthy volunteers was obtained from the Institutional Review Boards of the University of Florida College of Medicine and the School of Medicine at Washington University in St. Louis. Permission to obtain whole blood from trauma patients was obtained from the Institutional Review Boards of the University of Rochester School of Medicine and Harborview Medical Center, University of

Washington. Blood was collected from two healthy human subjects into heparinized blood collection tubes by personnel trained in phlebotomy using sterile technique on repeated occasions over a 12-mo period. A total of five replicate samples were obtained: four from a single subject and the fifth from the second healthy subject. At each donation, the blood was divided into four aliquots (Fig. 1). *Staphylococcus* enterotoxin B (SEB; Sigma Fine Chemicals, St. Louis, MO), at 2 µg/ml, was added to two of the four aliquots and incubated at 37°C for 2 h while the remaining two aliquots were processed immediately. One of the unstimulated and one of the SEB-stimulated aliquots of blood were transferred to PAXgene tubes, and RNA was isolated according to the manufacturer's instructions.

The remaining two aliquots were centrifuged at 500 g and the buffy coat layer was removed. This fraction contains the leukocyte populations as well as contaminating thrombocytes. Residual erythrocytes were lysed with 15 ml of EL Buffer (Qiagen, Valencia, CA) for 15 min at 4°C, and the leukocyte-rich fraction was collected by centrifugation. After a second wash in EL Buffer, the cells were collected again, and total cellular RNA was extracted using a commercial RNA purification kit (RNeasy; Qiagen, Valencia, CA). RNA quality was determined with an A₂₆₀/A₂₈₀ ratio and capillary electrophoresis on an Agilent 2100 Bioanalyzer automated analysis system (Agilent Technologies, Palo Alto, CA). Biotinylated cRNA synthesis was generated with 10 µg of total cellular RNA based on the protocol outlined by Affymetrix (Santa Clara, CA), with few modifications. cRNA was both analyzed on the Agilent 2100 Bioanalyzer and hybridized onto U95Av2 or U133A oligonucleotide arrays (Affymetrix), stained, and washed according to an Affymetrix protocol. Microarray data can be accessed through the Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) through the accession number GSE1343.

Blood samples were also collected from eight subjects who had been admitted to the hospital for major blunt trauma or burn injury (Table 1). In this case, ~20 ml of whole blood was collected from a

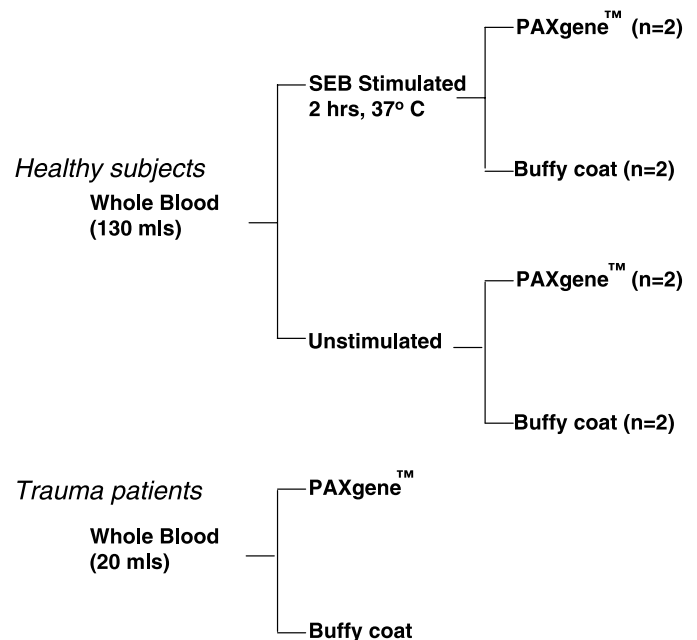


Fig. 1. Study design for the blood collection protocols. In healthy subjects, 130 ml of whole blood was collected and aliquoted into two fractions, one for ex vivo stimulation with *Staphylococcus* enterotoxin B (SEB), and the second processed further immediately. The two fractions were then divided and processed according to either the buffy coat or PAXgene protocol, yielding two replicates for each sample. The process was repeated a total of five times in the two healthy subjects. From trauma patients, 20 ml of blood was drawn a single time and divided into two aliquots that were processed simultaneously.

Table 1. Trauma patient characteristics, percent present calls, and correlation coefficients among replicates and between blood collection protocols

Subject ID	Injury Severity Score	Diagnosis
Subject 1	18	28-yr-old male 9 days post blunt trauma.
Subject 2	34	71-yr-old female 3 days post blunt trauma.
Subject 3	19	19-yr-old male 3 days post blunt trauma.
Subject 4	NA	48-yr-old male 5 days post 22% body surface area, full thickness burn
Subject 5	29	24-yr-old male 7 days post blunt trauma
Subject 6	22	35-yr-old female 3 days post blunt trauma
Subject 7	13	21-yr-old male 4 days post blunt trauma
Subject 8	29	48-yr-old male 3 days post blunt trauma

	Percent Present Calls (n = 8)	CC Between Different Subjects (n = 8)	CC Between RNA Isolation Methods (n = 8)
PAXgene	28.5 ± 3.7*	0.850	0.766
Buffy coat	43.2 ± 2.4	0.931	

Values are means ± SE. The percent present calls were identified using Affymetrix Microarray Suite v. 5 (MAS 5). Pearson correlation coefficients (CC) were performed on samples from the same blood draw (PAXgene vs. buffy coat fraction) and from the different individuals with the same sampling technique using the entire U133A data set (22,281 probe sets). * $P < 0.001$ vs. the corresponding buffy coat analysis, by Student's *t*-test.

subject. The blood sample was divided into two aliquots, and one was processed according to the leukocyte protocol described above, while the other was collected in the PAXgene system and processed accordingly (Fig. 1). Blood samples from trauma patients were not stimulated *ex vivo* with SEB.

Because the principal difference between the two techniques is the inclusion of RNA from the erythrocyte fraction, we investigated whether an overabundance of hemoglobin mRNA might explain the differences in expression between whole blood and leukocyte generated samples. Additional RNA was isolated from whole blood from the healthy subjects using the PAXgene blood collection scheme. Degradation of multiple hemoglobin mRNA species in the samples was accomplished using RNase H and specifically designed primers for nine common hemoglobin genes¹ on four donor samples. Briefly, 5 µg of total cellular RNA was incubated in 10 mM Tris·HCl, pH 7.6, 20 mM KCl with 10 µM of oligonucleotide primers at 70°C for 5 min. The samples were cooled to 4°C, and 2 U of RNase H (New England Biolabs), along with 20 U of SUPERase Inhibitor (Ambion), was added. The buffer conditions were adjusted to 55 mM Tris·HCl, 85 mM KCl, 3 mM MgCl₂, and 10 mM dithiothreitol, and the samples were incubated at 37°C for 15 min. Immediately following the incubation, the samples were again cooled to 4°C and 1 µl of 0.5 M EDTA was added to stop the RNase H digestion. The samples were then repurified using the RNeasy Mini Protocol for RNA Cleanup (Qiagen), according to the manufacturer's specifications and including the optional DNase treatment.

¹Oligonucleotide primers for the principal hemoglobin species were synthesized at the Stanford Genome Technology Center. The following sequences were used: 5'-GTGATCTTAGCAGAATAGATTTATTATTGATTGCTTGCAG-3' hemoglobin-γG; 5'-GTGATCTCTCAGCAGAATAGATTTATTATTG-TATTGCTTGCAG-3' hemoglobin-γA; 5'-GTTGAGCTGAACATCTTATT-AGGCAGAAGCCATACCC-3' hemoglobin-δ; 5'-TTAGGCAGAATCCA-GATGCTCAAGGCCCTTCATAATATCC-3' hemoglobin-β; 5'-CCGC-CCAATCAGACTTTATTCAAAGACCACGGGGGTAC-3' hemoglobin-α1; 5'-TGTTGGCAGATTCCGGGACAGAGAGAACCAGGCACACAC-3' hemoglobin-α2; 5'-CTTCGTTCAATTGGTTTATTGGCGCATTACGCGGTA-AGTGG-3' hemoglobin-ζ; 5'-GCTCCTGGGAGAGGCTTACTCAAACACGGGGAAGGTCGC-3' hemoglobin-θ1; 5'-GATTACTGAAGAAAATGTACTTTATTAACAGAAGGCTTTCTC-3' hemoglobin-ε1.

Statistical approaches. To test the validity of the null hypothesis that the two RNA isolation methods were equivalent, a balanced subset of microarrays was used from both the healthy subjects and the trauma patients. After array normalization and filtering the universally "absent" probe sets with Microarray Suite ver. 5.0 (MAS 5.0, Affymetrix), the expression levels of the remaining probe sets were square root transformed to stabilize the "within gene" variance. Differences in the concordance and the percent present calls between samples generated from the whole blood and buffy coat methodologies were evaluated using the Student's *t*-test.

The hybridization signal intensities from both healthy subjects' and trauma patients' blood collected with the leukocyte and PAXgene blood systems were examined by hierarchical cluster analysis using Cluster² and TreeView (3). In addition, the hybridization signal intensities of the genes from healthy subjects (unstimulated and stimulated *ex vivo* with SEB) were analyzed using BRB Array Tools 2.1³ (2) to identify genes at the $P < 0.001$ level of significance that differentiated among the four treatment classes (buffy coat SEB stimulated, buffy coat unstimulated, PAXgene stimulated, PAXgene unstimulated). The ability of genes identified from the healthy subjects in predicting treatment class was assessed using "leave-one-out" cross-validation using each of four methods of class prediction: one-nearest neighbor, three-nearest neighbors, linear discriminate analysis, and nearest centroid analysis.

Each experiment in healthy subjects from five replicates yielded four differences: two independent estimates of the effect of SEB stimulation as measured by each RNA isolation method. To determine whether the changes in gene expression with one of the RNA extractions methods were noisier than the other, the intraclass correlation coefficient, or reliability (4), of each method was computed using a variance components model. The permutation distribution of the ratio of the two reliabilities was computed to test the significance of the magnitude of the leukocyte/whole blood reliability ratio.

RESULTS AND DISCUSSION

Although gene expression analyses offer an unprecedented opportunity to explore the integrated genome-wide response to injury and disease, their use in hospitalized patients has been limited by the absence of a critical evaluation of the optimal methods for the isolation of nucleic acids from human tissues, as well as their analysis and statistical evaluation. Considerable effort has been directed at optimizing the gene expression platform, as well as a detailed analysis of the variation associated with the synthesis of a hybridization target (16). But little effort has focused on the importance of blood collection and RNA isolation schemes on subsequent gene expression profiles. Prior to initiating large multicenter studies in critically ill trauma and burn patients, we conducted a series of experiments to evaluate the reliability, signal-to-noise ratio, and variance associated with the analysis of gene expression profiles from human blood. Such studies are a critical first step toward establishing a blood collection and RNA isolation platform that will be able to dissect changes in gene expression that occur in response to severe trauma and burn injury and can be used for class identification, prediction, and, more importantly, to elucidate the underlying biological mechanisms re-

²These statistical programs (Cluster and TreeView) are available for download from the Eisen Laboratory web site (<http://rana.lbl.gov/EisenSoftware.htm>).

³The BRB Array Tools ver. 2.1 software program was developed by Drs. Richard Simon and Amy Lam, from the Department of Bioinformatics at the National Cancer Institute, NIH. The software is available directly from the investigators' web site (<http://linus.nci.nih.gov/BRB-ArrayTools.html>).

sponsible for outcome from severe trauma and injury. To that end, samples were obtained from healthy subjects and trauma patients, and results were compared between RNA extraction protocols using either a traditional leukocyte (buffy coat) isolation or a whole blood RNA collection system. To simulate the changes in gene expression that would be anticipated following microbial infection, blood samples from healthy subjects were stimulated ex vivo with SEB. Our concern was whether the reproducibility, yield, and the quality of the isolated total RNA from either of the two protocols would be acceptable to meet our criteria for a high-resolution clinical study subsequently conducted in critically ill patients.

As shown in Table 2, Affymetrix U95A microarrays hybridized with cRNA generated from the whole blood collection system (PAXgene) from healthy subjects had consistently fewer probe sets whose signal intensity was greater than that of the background noise of the array compared with leukocyte samples ($P < 0.01$, by Student's t -test). This was also seen with U133A microarrays performed on RNA samples obtained from trauma patients ($P < 0.01$, by Student's t -test; Table 1). However, the reproducibility of the response at the microarray level was extremely high in samples obtained from both the leukocyte and whole blood RNA collection system from healthy subjects (Table 2). As expected, there was more variation in gene expression among the different trauma subjects (Table 1). Pearson correlation coefficients obtained from two replicates at each blood sampling interval from the healthy subjects (both with and without SEB stimulation) averaged 0.989 ($n = 8$) and 0.992 ($n = 8$), respectively, for the leukocyte isolation and whole blood collection systems. Similarly, correlation coefficients in replicates obtained from the same healthy subject from different blood sampling intervals were always greater than 0.965. Thus the signal intensities with the Affymetrix GeneChip platform and the apparent gene expression results were highly concordant when performed on blood or leukocyte samples from healthy subjects, and the response in the same healthy subject over time was constant.

Table 2. Percent present calls, CC, CV, and reliability from SEB-stimulated whole blood and leukocyte preparations from healthy subjects

	Percent Present Calls	CC Within Replicates	CC Between Replicates	CV	Reliability
Buffy Coat					
Without SEB	41.7 ± 4.2	0.992	0.972	0.32	0.749
With SEB	40.0 ± 5.5	0.986	0.968	0.33	
PAXgene					
Without SEB	29.6 ± 4.0*	0.991	0.966	0.37	0.461†
With SEB	27.4 ± 9.1*	0.992	0.963	0.46	

Values are means ± SE. The percent present calls were the identified using Affymetrix MAS 5. Pearson correlation coefficients were performed on replicate samples from the same blood draw (within replicates) and from the same individual on different blood draws (between replicates) using the entire U95Av2 data set (12,625 probe sets), whereas the coefficient of variation (CV) and the reliability were calculated from 7,496 probe sets after elimination of those probe sets marked "absent" on all 36 arrays. For correlation coefficients, the one PAXgene outlier was removed prior to analysis (see text). * $P < 0.001$ vs. the corresponding buffy coat analysis, by Student's t -test. † $P = 0.002$ is the rank of the reliability ratio in the permutation distribution. SEB, *Staphylococcus enterotoxin B*.

Similarly, we evaluated whether the two techniques were equivalent in terms of their ability to detect changes in gene expression in response to ex vivo stimulation with SEB. In this case, we examined whether one of the RNA isolation methods exhibited a greater signal-to-noise ratio than the other, using the intraclass correlation coefficient. Significant differences were observed between the whole blood and leukocyte RNA isolation methods. The reliability ratios for leukocyte and whole blood samples were 0.749 and 0.461, respectively (Table 2). The rank of the reliability ratio in the permutation distribution was 0.998, confirming that the two techniques were not equivalent, and the reliability of the leukocyte method was significantly greater than it was for whole blood ($P = 0.002$).

These differences in the ability of the two RNA isolation systems to detect changes in gene expression in response to SEB stimulation are visually presented in the cluster analyses contained in Fig. 2. After array normalization and initial expression filtering, 2,746 probe sets were identified at the $P < 0.001$ level as being able to discriminate between the four treatment groups (buffy coat and PAXgene, unstimulated and SEB stimulated) using BRB Array Tools v2.1. Hierarchical clustering of the variance normalized expression profiles demonstrated that the RNA isolation method (whole blood vs. leukocyte) was the major response variable, not ex vivo stimulation with SEB. Cross validation studies correctly identified the class label for each array in both groups. Using the leukocyte isolation procedure, 943 probe sets exhibited changes in expression with ex vivo SEB stimulation at the $P \leq 0.001$ level. Only 303 probe sets discriminated between SEB-stimulated and unstimulated whole blood using the whole blood RNA isolation technique. We found that 254 probes sets were identified with significant differences in hybridization signal intensities after ex vivo SEB stimulation with both methods [180 (70.9%) upregulated and 74 (29.1%) downregulated].

One of the arrays on the PAXgene side of the experiment appeared to be an outlier based on the low number of probe sets called "present" by MAS 5.0. Inspection of the input RNA by the Agilent 2100 Bioanalyzer suggested that the RNA was partially degraded prior to target (biotinylated cRNA) preparation. Using the recommendations of the Tumor Analysis Best Practices Working Group, published recently in *Nature Reviews Genetics*, this sample, but none of the others generated in this analysis, should have been discarded prior to cRNA synthesis (15). We therefore removed this array and recomputed the number of significant probe sets. For balanced comparison, we also removed corresponding arrays from the buffy coat side of the experiment. When these adjustments were made, buffy coat yielded 718 significant probe sets responsive to SEB treatment, compared with 354 probe sets for PAXgene (at the $P < 0.001$ level). Although there were a number of common probe sets with both techniques whose apparent expression was similarly changed by ex vivo SEB stimulation, there was a twofold difference in the number of significant probe sets whose change in expression in response to ex vivo SEB stimulation was dependent upon the RNA isolation protocol.

This latter observation has important ramifications for the interpretation of results from studies employing different RNA stabilization and isolation strategies. One of the principal

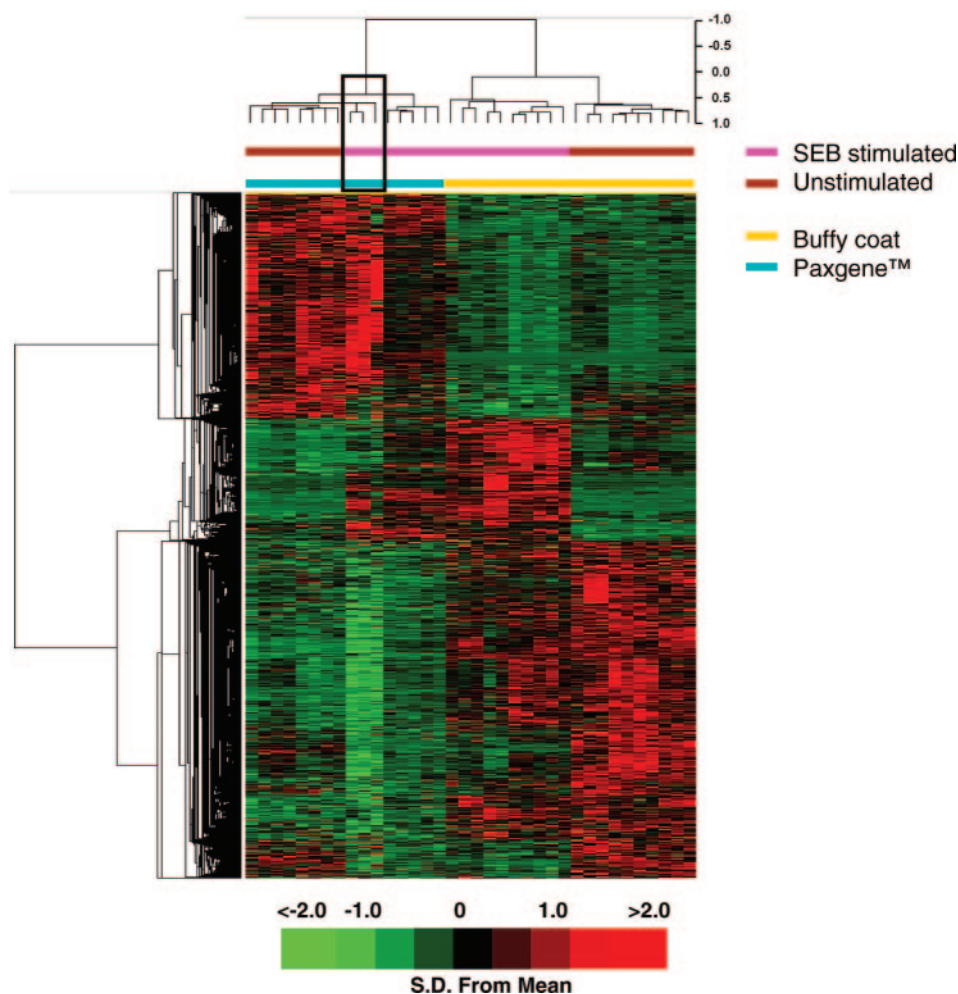


Fig. 2. Clustering of 2,746 probe sets, significant at $P \leq 0.001$, that discriminated among the four experimental classes (buffy coat unstimulated, buffy coat stimulated, PAXgene unstimulated, and PAXgene stimulated). Microarray analyses were initially filtered for present calls. Based on hierarchical analysis, the major division was the RNA isolation method. Subdivisions within leukocyte distinguished between SEB-stimulated and unstimulated samples. Three arrays from the whole blood SEB-stimulated samples cluster with the whole blood unstimulated samples (in box).

conclusions that can be reached from the present study is that evaluating gene expression patterns in whole blood or leukocytes obtained from different RNA isolation protocols will be problematic, and the method of RNA isolation needs to be considered a critical variable in the design of the experiment. In addition, the results suggest that different RNA stabilization and isolation strategies will introduce varying amounts of analytical noise into the system.

The whole blood collection systems (such as PAXgene) offer a number of potential advantages that make them highly attractive for multicenter clinical studies, the primary one being their ease of use. In fact, these techniques have been promulgated in a number of recent clinical studies looking at blood gene expression patterns based on microarray analyses or RT-PCR (9, 10). We have shown, however, that there is increased noise and reduced responsiveness in the gene expression profiles derived from whole blood compared with a leukocyte isolation protocol. Analysis of the gene expression profiles from the whole blood preparations revealed that the three most abundantly expressed genes were for hemoglobin- β and hemoglobin- $\alpha 1$ (Table 3). In these cases, the signal intensity for these highly abundant genes was at the level of probe saturation, so it was difficult to estimate how much greater they were in the whole blood than in the leukocyte isolation protocols. The relative expressions of several less abundant eryth-

rocyte- or reticulocyte-specific genes, (selenium-binding protein, erythrocyte membrane band proteins 4.3 and 4.9, hemoglobin- δ and - γ), however, were 19–71 times higher in the whole blood than in the leukocyte preparations. Furthermore, the expression of these reticulocyte-specific genes in the whole blood system did not significantly change in response to SEB stimulation.

A comparative analysis of amplified cRNA between leukocyte and whole blood samples using the Agilent 2100 Bioanalyzer revealed a predominant band at ~ 680 bp in the whole blood samples (Fig. 3), a strong indication that high-abundant mRNAs present in that position were members of the multi-gene family of hemoglobin. This is consistent with the primary difference between the two methodologies being the removal of erythrocytes from the buffy coat fraction. Centrifugation of whole blood at 500 g results in the concentration of leukocytes and thrombocytes into the interfacial band, while contaminating erythrocytes are subsequently lysed. Both preparations would therefore include RNA from the leukocyte population as well as from thrombocytes, which may contain an appreciable amount of mRNA (5). The primary difference between these two methodologies, therefore, is the residual mRNA associated with erythrocyte or reticulocytes. To decipher the precise etiology of this band, methodologies were developed to specifically eliminate the principal hemoglobin genes from the

Table 3. Relative expression of reticulocyte- and erythrocyte-specific genes obtained from whole blood and leukocyte preparations

Probe Set	Gene	Relative Signal Intensity	Fold Change	
			PAXgene vs. Buffy coat	SEB vs. no SEB
37405_at	selenium binding protein	16,414	81.5	0.8
36871_at	erythrocyte membrane protein band 4.2	2,062	71.3	0.8
33516_at	hemoglobin- δ	9,367	54.8	0.8
38585_at	hemoglobin- γ	48,976	22.1	0.8
37192_at	erythrocyte membrane protein band 4.9	29,398	19.1	0.9
39839_at	cold shock domain protein A	6,176	15.2	0.8
40850_at	FK binding protein 8	7,794	9.8	1.0
31687_f_at	hemoglobin- β	90,678	2.3	1.1
31525_s_at	hemoglobin- $\alpha 1$	82,488	2.1	1.1
32052_at	hemoglobin- β	87,987	1.7	1.1

The relative abundance of expression of genes from the Affymetrix U95Av2 GeneChip in samples obtained from a whole blood (PAXgene) and a leukocyte preparation (buffy coat). The relative signal intensity is presented for the PAXgene, unstimulated group, and is obtained from the Affymetrix U95A GeneChip following normalization of signal intensity with MAS 5. The fold change represents the difference in expression between PAXgene and buffy coat, and between SEB-stimulated (SEB) and unstimulated (no SEB) samples.

whole blood RNA preparations using hemoglobin-specific antisense oligonucleotides and digestion of the RNA sample with RNase H. Degradation of the hemoglobin mRNAs eliminated the single cRNA species seen in the whole blood preparations and generated electrophoretic patterns that were more comparable, but not identical, to those seen with leukocyte preparations (Fig. 3). More specifically, whole blood samples in which the major hemoglobin species were degraded with RNase H treatment now clustered separately from the original whole blood samples (Fig. 4) and in a subnode distinct from the leukocyte samples. This latter finding is consistent with the conclusion that erythrocytes or reticulocytes, and other non-leukocyte sources, contribute an appreciable number of mRNA species in the whole blood collection system, but simply removing the over-abundant hemoglobin mRNA species does

not result in a response pattern identical to that seen from leukocytes alone.

To examine whether differences in gene expression between the two collection methodologies (PAXgene vs. buffy coat) were also evident in our target trauma patient population, blood was collected from eight trauma or burn patients, divided into two aliquots and processed with the two techniques. As shown in Fig. 5, hierarchical cluster analysis of the hybridization signals from the probes set showed that the primary determinant of gene expression was the method of RNA isolation and not differences among the trauma patients. Gene expression patterns clustered not depending upon the individual patient, but due to the method of RNA isolation. Furthermore, as shown in Table 1, concordance in gene expression among the trauma patients

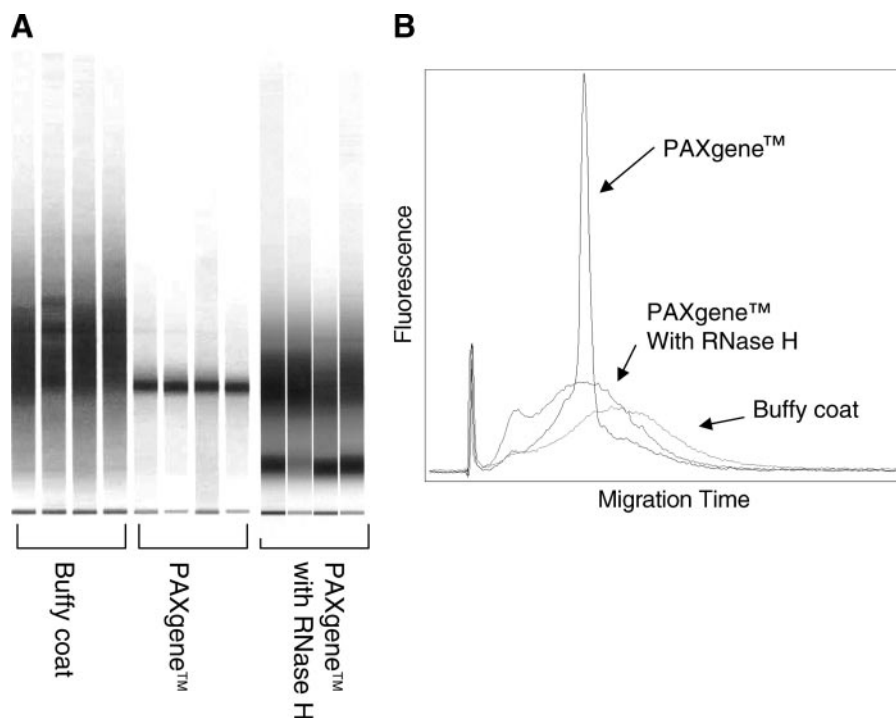


Fig. 3. Capillary electrophoretic patterns of cRNA generated from either whole blood or leukocyte preparations. A: pseudogel presentation of Agilent 2100 Bioanalyzer output of cRNA generated from four samples obtained from buffy coat and whole blood preparations (PAXgene) with and without RNase H pretreatment. B: the electrophoretic patterns between whole blood and leukocyte preparations in a representative sample are differentiated by a high-abundant species seen in the whole blood preparation. This species is effectively degraded by RNase H treatment.

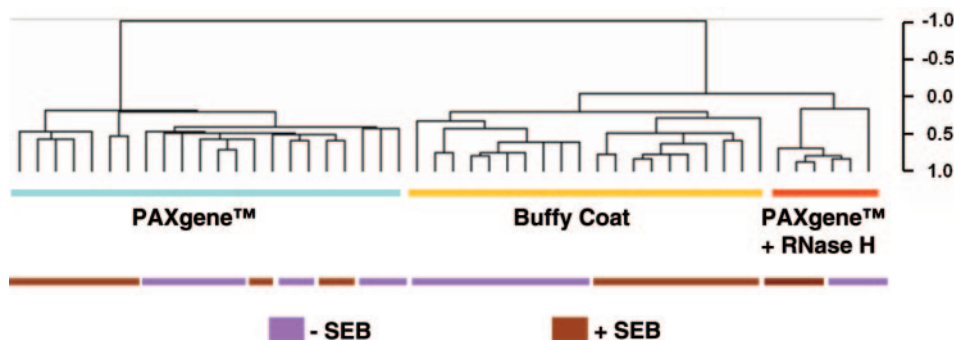


Fig. 4. Clustering of probe sets that discriminated among six experimental classes (buffy coat unstimulated; buffy coat stimulated; PAXgene unstimulated; PAXgene stimulated; PAXgene unstimulated, RNase H treated; and PAXgene stimulated, RNase H treated) in blood obtained from a single healthy volunteer. Again, the major division is the RNA isolation method. RNase H-treated whole blood now clusters separately from the original PAXgene, but in a subnode also separate from the buffy coat samples.

was significantly higher with the leukocyte preparation than with PAXgene, consistent with a greater amount of analytical noise in these measurements (0.931 vs. 0.850, $P < 0.05$ by Student's t -test).

Conclusions and Recommendations

The widespread use of gene expression analyses has been limited by the lack of a critical evaluation of the methods used to extract nucleic acids from human tissues. Such studies are an essential requisite for establishing blood collection and RNA isolation protocols that can discriminate changes in gene expression for class prediction and the identification of underlying biological mechanisms in injury and disease. The findings from this study demonstrate that whole blood and circulating leukocyte isolation protocols both generate high-quality RNA that can yield highly reproducible results when used with the Affymetrix GeneChip system. However, the method used to

isolate RNA has a significant impact on the gene expression profiles obtained from human whole blood or circulating blood leukocytes. This finding has important ramifications for the interpretation of results from clinical studies employing different RNA stabilization and isolation strategies for the evaluation of gene expression patterns in blood. Evaluating gene expression patterns in whole blood or leukocytes obtained from different RNA isolation protocols will be difficult, and the method of RNA isolation needs to be considered a critical variable in the design of the experiment.

These whole blood collection systems, like PAXgene, offer a number of technical advantages that make them highly attractive for multicenter clinical studies. However, there appear to be limitations to their use, including increased noise and reduced responsiveness in the gene expression profiles derived from whole blood compared with a leukocyte isolation protocol. Such findings will have important implications for

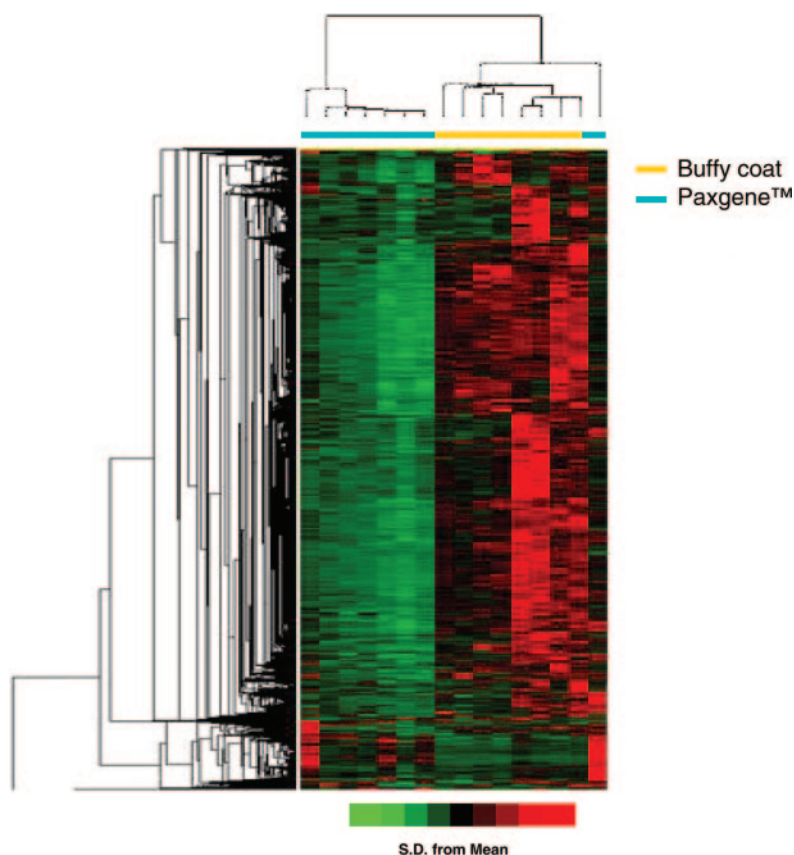


Fig. 5. Hierarchical clustering of the top 50% of all the variance normalized probe sets ($n = 6,718$) from the eight trauma/burn subjects processed with both the buffy coat and PAXgene protocols using the Affymetrix U133A GeneChip. GeneChip analyses were initially filtered for present calls (see MATERIALS AND METHODS), reducing the number of probe sets to 13,436, and then the top half of the probe sets with the greatest variance were hierarchical clustered. Based on the hierarchical analysis, the major division was the RNA isolation method and not the individual patients.

the design of future clinical studies and the increased need for replicates. This reduced signal-to-noise ratio and the reduced responsiveness of whole blood collection systems, such as PAXgene, will require greater numbers of study patients to identify genes whose expression may discriminate an injury response and outcome. Ultimately, the decision on the optimal RNA isolation protocol for whole blood will be based on both theoretical and practical concerns.

ACKNOWLEDGMENTS

As noted in author line, there are additional participating investigators in the Large-Scale Collaborative Research Program titled "Inflammation and the Host Response to Trauma": Paul E. Bankey, Timothy R. Billiar, Steven E. Calvano, Irshad H. Chaudry, Chuck Cooper, Bradley Freeman, Richard L. Gamelli, Nicole S. Gibran, Brian G. Harbrecht, Wyrta Heagy, David M. Heimbach, David N. Herndon, Jureta W. Horton, John Lee Hunt, Jeffrey Johnson, James A. Lederer, Tanya Logvinenko, Stephen F. Lowry, John A. Mannick, Bruce A. McKinley, Joseph P. Minei, Ernest E. Moore, Frederick A. Moore, Avery B. Nathens, Grant E. O'Keefe, Laurence G. Rahme, Daniel G. Remick, Jr., Michael B. Shapiro, Robert L. Sheridan, Geoffrey M. Silver, Richard D. Smith, Scott Somers, Gregory Stephanopoulos, Mehmet Toner, H. Shaw Warren, Michael A. West, Steven E. Wolf, Martin Yarmush, and Vernon R. Young.

GRANTS

This work was supported National Institute of General Medical Sciences Grant U54-GM-62119-02, for the "Injury and the Host Response to Inflammation" Large-Scale Collaborative Research Program.

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