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Microarray kit analysis of cytokines in blood product units and segments

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Abstract

BACKGROUND—Cytokine concentrations in transfused blood components are of interest for some clinical trials. It is not always possible to process samples of transfused components quickly after their administration. Additionally, it is not practical to sample material in an acceptable manner from many bags of components before transfusion, and after transfusion, the only representative remaining fluid of the component may be that in the “segment,” as the bag may have been completely transfused. Multiplex array technology allows rapid simultaneous testing of multiple analytes in small volume samples. We used this technology to measure leukocyte cytokine levels in blood products to determine (1) whether concentrations in segments correlate with those in the main bag, and thus, whether segments could be used for estimation of the concentrations in the transfused component; and (2) whether concentrations after sample storage at 4°C for 24 hrs do not differ from concentrations before storage, thus allowing for processing within 24 hrs, rather than immediately after transfusion.

STUDY DESIGN AND METHODS—Leukocyte cytokines were measured in the supernatant from bags and segments of leukoreduced red blood cells, non-leukoreduced whole blood, and leukoreduced plateletphereses using the ProteoPlex Human Cytokine Array kit (Novagen).

RESULTS—Cytokine concentrations in packed red blood cell and whole blood, or plateletphereses stored at 4°C did not differ between bag and segment samples (all $p > 0.05$). There was no evidence of systematic differences between segment and bag concentrations. Cytokine concentrations in samples from plateletphereses did not change within 24 hrs storage at 4°C.

CONCLUSION—Samples from either bag or segment can be used to study cytokine concentrations in groups of blood products. Cytokine concentrations in plateletphereses appear to be stable for at least 24 hrs of storage at 4°C, and, thus, samples stored with those conditions may be used to estimate the cytokine concentrations of the component at the time of transfusion.

Keywords

Cytokines; transfusion reaction; blood transfusion; red blood cell transfusion; platelet transfusion; whole blood transfusion; multiplex assays

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Conflict of Interest: None

Introduction

Transfusion medicine specialists have long had an interest in changes that occur during blood storage and how the changes may affect the recipient of the blood product. This interest continues to grow, and has led to an initiative from the National Heart Lung and Blood Institute in 2008 to study the “Immunomodulatory, Inflammatory, and Vasoregulatory Properties of Transfused Red Blood Cells as a Function of Storage Time”. Among biologic response modifiers in stored blood, leukocyte derived cytokines are of particular interest because of their well-known association with immune and inflammatory transfusion reactions.¹⁻⁶ Previous studies have mostly used Enzyme Linked Immunosorbent Assays (ELISA) for single analytes, which are not suited to the testing of large numbers of cytokines of large numbers of samples. Microarray technology allows rapid simultaneous testing of multiple analytes using very small volumes of samples, and, to our knowledge, has rarely been used to test for cytokines in blood products. Cytokine concentrations of transfused blood components are of interest for clinical trials of transfusion-related lung injury (TRALI), as they are implicated in some hypotheses of the etiology of TRALI.⁷⁻⁸ Also of interest to the transfusion medicine community are studies of leukocyte-derived cytokines in the evaluation of new methods for collection of blood components,⁹ evaluation of pathogen inactivation of platelet concentrates,¹⁰ and for investigation as a possible quality control measure of the adequacy of leukoreduction.¹¹ As a practical matter, at least two logistic issues impede the estimation of cytokine concentrations of transfused components. First, there may be inadequate material in the component bag after transfusion, and it is impractical to sample, in an acceptable manner, material from large numbers of bags of components before they are transfused. Second, it is not always practical to prepare the sample for analysis immediately after transfusion. However, it is not known whether the blood sample in the segment attached to the main bag could serve as a surrogate sample of the actual material in the bag, which could significantly facilitate screening of blood donor units. Additionally, there are few data describing whether the cytokine concentrations of samples are affected by length of storage time and temperature at 4°C. Therefore the aims of this study were, using antibody a commercial microarray kit, to determine (1) whether cytokine concentrations are similar in bags and segments in units of non-leukoreduced whole blood, leukoreduced red blood cells and leukoreduced plateletphereses, and (2) whether cytokine concentrations change during 24 hour storage of plateletphereses samples at 4°C. The answers to these questions would be of substantial assistance for our clinical studies of TRALI.

Materials and Methods

A. Blood products studied

Discarded blood products (units) were collected after transfusion or within a week of outdating. In-date products were not required, as the purpose of the study was not to study the absolute concentrations of cytokines in transfusable products, but rather to compare concentrations in segments and bags at the same time point of identical conditions. Blood products used for the study included 10 units of 39-42 (median 40) day old, non-leukoreduced whole blood in CPDA-1 anticoagulant, 14 units of 10-42 (median 31.5) day old pre-storage leukoreduced packed red blood cells in ADSOL anticoagulant, and 10 units of 4-9 (median 5) day old leukoreduced plateletphereses, processed for analyses within 24 hours of collection. Supernatant plasma samples tested for leukocyte cytokines were prepared in the same way for all the blood products, by centrifugation at 3000 rpm for 10 minutes at 4°C. Aliquots of plasma were then removed and stored at -20°C until they were assayed.

B. Study Design

Comparison of cytokines in bags and segments of packed red blood cells and whole blood

To determine whether cytokine concentrations in segments and bags were similar, 14 units of pre-storage leukoreduced packed red blood cells and ten units of non-leukoreduced whole blood were studied. Residual blood from the units and segments was collected into two separate tubes. After centrifugation of the samples at 3000 rpm for 10 minutes at 4°C, 30 µl aliquots of supernatant were removed from each sample and stored at -20°C. Leukoreduced plateletpheresis units were also studied as described below.

Cytokine concentrations in plateletphereses and effect of 24 hour storage: To determine whether cytokine concentrations of samples change with 24 hrs of storage at 4°C, cytokine concentrations were compared in samples, after removal from bags and segments, and after storage for 24 hours at 4°C. Ten units of leukoreduced plateletphereses (four to nine days old; median 5 days) were studied. For each plateletpheresis unit, three samples of 200 µl were from both the bag and segment. One sample was immediately centrifuged at 3000 rpm for 10 minutes at 4°C, and then a 30 µl aliquot of plasma was withdrawn and stored at -20°C. The remaining two samples were stored at 4°C. At four hours, one tube stored at 4°C was centrifuged, and 30 µl plasma aliquots were removed and stored at -20°C. At 24 hours the second tube was centrifuged, and 30 µl aliquots of plasma withdrawn and stored as for the previous samples.

C. Measurement of cytokines

Cytokine concentrations were measured using the commercial microarray kit, ProteoPlex Human Cytokine Array manufactured by Novagen (EMD Biosciences, Inc.; Madison, WI). The slide chip is based on an ELISA. On each slide, there are ten wells for samples. In each well, a 50µl sample can be tested in quadruplicate for 12 cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, GM-CSF, IFN-γ, TNF-α. Two slides (20 samples) were tested in parallel per day. Standard curves were determined on each chip and both bag and segment samples from the same unit were always assayed on the same slide. Each sample was assayed in quadruplicate. The assay took approximately 6 hours, 3.5 hours of which was incubation time. A single investigator (R.Y.) performed all assays. The slides were shipped at 0°C to Novagen for analysis. Pixels with density values greater than four medians of absolute deviation removed from the median value were ignored, and the remaining pixel density values averaged to yield a spot density value. An automated computerized program (at Novagen) detected and removed “outlier” spots when the density value of one of the four spots of a sample was five standard deviations or more from the average of the four spots (owing to surface or assay variability).

Statistics

The minimum detectable cytokine concentration (5 pg/ml) was assigned to those cytokine concentrations that were below the minimal detectable concentration. Nearly all data sets did not have normal distribution. Therefore, all analyses were conducted with data after log transformation of all measured values. Data are reported as mean (95% CI). Individual cytokine concentrations from segments and bags were compared by a 2-tailed, paired t-test. Linear regression and analyses using the method of Bland and Altman¹² were used to compare combined data for cytokine concentration from segments and bags for all cytokines for each blood component. Cytokine concentrations for samples from platelets stored for 0, 4, and 24 hours were compared by analysis of variance with repeated measures. Statistical significance was accepted at $p < 0.05$.

Results

The coefficients of variation (cv) of the analyses were generally less than 10%.

Comparison of cytokine concentrations in segments with bags of blood components

Too few samples from leukoreduced packed red blood cells had measurable concentrations to permit valid analyses, except for IL-7 and IL-8. Concentrations for both IL-7 and IL-8 did not differ between segments and bags (Fig 1; $p = 0.72$ and 0.79 respectively).

In non-leukoreduced whole blood, concentrations for all cytokines did not differ between segments and bags (Fig 2; range of p values: $0.10 - 0.92$); for IL-1 α there were an insufficient number of samples with a measurable concentration of this cytokine.

Concentrations for all cytokines in plateletphereses did not differ between segments and bags (Fig. 3; range of p values $0.31 - 0.97$).

Erythrocyte products and plateletphereses showed significant linear correlation between cytokine concentrations from segments and bags (Figs. 4 and 5; r^2 erythrocyte products 0.95 , plateletphereses 0.97), with slopes not different from 1 (erythrocytes 1.01 ; plateletphereses 0.98). Analyses using the method of Bland and Altman did not demonstrate a systematic bias (Figs. 4 and 5; slopes not significantly different from zero; erythrocyte products: slope 0.04 , $p=0.69$; plateletphereses: slope 0.001 , $p=0.89$).

Cytokine concentrations in plateletphereses and effect of 24 hour storage

The standard curves for two plateletpheresis samples were not sufficiently linear (as determined by a blinded observer); these samples were eliminated from further consideration.

Mean cytokine concentrations (95% C.I.) in the main plateletphereses bags were: IL-1 α 25.2 pg/ml (21.3-29.1), IL-1 β 52.6 pg/ml (49.1-56.1), IL-2 30.4 pg/ml (27.1-33.8), IL-4 26.6 pg/ml (23.1-30), IL-6 33.7 pg/ml (30.7-36.7), IL-7 188.4 pg/ml (185.4-191.4), IL-8 66.5 pg/ml (64.1-68.8), IL-10 29.6 pg/ml (25.8-33.4), IL-12 107.9 pg/ml (102.2-113.7), GM-CSF 46.8 pg/ml (42.5-51.2), IFN- γ 173.8 pg/ml (168.4-179.2), and TNF- α 81.4 pg/ml (77.7-85.2). Storing plateletphereses for 24 hours at 4°C did not change concentrations of any individual cytokine, nor when all cytokines were considered cumulatively ($p=0.10$).

Above analyses repeated without assigning the minimum detectable cytokine concentration to those samples that were below the minimum detectable concentration did not produce different results.

Discussion

Our study demonstrates that cytokine concentrations did not differ between bag and segment samples from the same unit of blood, and the concentrations in bag and segment are strongly correlated, without a systematic bias. Thus, investigators can use either blood in the segment or the main bag when examining cytokines in blood donor products. Our study also found no changes in cytokine concentrations of samples stored for 24 hours at 4°C. Thus, samples for studies can be stored for 24 hours at 4°C prior to testing. The strong correlation between concentrations in segments and bags for plateletpheresis units is expected, because blood in the segments is in a space contiguous with that in the bag, i.e. segments are not crimped and sealed off from the bag. Thus, multiple samples of blood in a contiguous space are likely to yield the same results. In contrast, segments in red blood cell and whole blood units are traditionally sealed off from the primary bag at the time of blood donation and collection, in

order to facilitate removal of blood for re-typing of the ABO group of the red cells. Hence the blood in segment and bag is not in a contiguous space, and yet, the cytokine concentrations correlated well.

Cytokine concentration differences between the segment and the bag may theoretically vary because residual leukocytes or platelets that produce the cytokines may be differentially distributed or may be more viable in the segment or bag. Nevertheless, our observations demonstrate a strong correlation, without systematic bias, of cytokine concentrations in segment versus the bag, allowing investigators ready access to samples from blood donor units without having to sample the unit directly, or incurring the costs of sampling from the bag with a device that maintains sterility.

Cytokines are soluble proteins or glycoproteins, which are produced by leukocytes, platelets and other cells, and act as chemical communicators between cells. Some of the functions of cytokines are known to be relevant to transfusion reactions. For example, febrile, non-hemolytic transfusion reactions are associated with stored cellular blood products, and are reduced by pre-storage leukoreduction or washing.⁹ According to The Cytokine Facts Book, IL-1 causes hypotension, fever, neutrophilia, and an acute phase response; IL-1 β is mostly secreted and is present in the plasma of stored platelets.¹ IL-6 regulates acute phase reactions and is also present in the plasma of stored platelets.¹ IL-8 is an inflammatory chemokine and attracts and activates neutrophils, and may contribute to the neutrophil priming activity of plasma in stored platelets.⁷ IFN- γ regulates nearly all phases of immune and inflammatory responses and enhances histocompatibility antigen expression on antigen presenting cells. We suggest that in theory, TRALI might be more likely or more severe if IFN- γ enhances expression of recipient histocompatibility antigens that are cognate to transfused donor antibody. In addition, TNF- α has been implicated in the development of acute respiratory distress syndrome.¹⁴

As expected, most leukocyte-reduced red blood cells did not have detectable cytokines, except for IL-7 and IL-8. It is not clear why these cytokines were routinely detected despite leukoreduction of the PRBC units. Leukocyte cytokine concentrations detected in the leukoreduced plateletphereses were similar¹¹ or higher¹⁰ than those in previous studies. We may have observed higher values because units in this study included outdated units, or perhaps some of the units were not adequately leukoreduced, or owing to our use of a different assay, that may be assessing different epitopes. Our study also found no changes in cytokine concentrations of samples stored for 24 hours at 4°C. Thus, samples for studies can be stored for 24 hours at 4°C prior to testing.

There were limitations with the cytokine chip assay. Results were not available for all cytokines in every sample because (1) the cytokine concentration was below the lower limit of detection of the assay especially in leukoreduced blood products; or (2) the cytokine concentration was too high at the dilution recommended by the manufacturer; or (3) for two samples the standard curve was inadequate. Although a number of cytokine concentrations were below the lower limit of detection of the assay (5 pg / mL), this did not alter the results, nor would any changes or differences in cytokine concentration below this lower limit likely have any clinical significance. Similarly, an inability to measure a few extremely high concentrations would also be without clinical import, as it would be sufficient to know that the concentration was extremely high, without knowing the exact value. Although data were incomplete, this study shows that microarray technology is potentially useful for the study of cytokines in blood products. Another potential limitation of our report is that shortly after completion of the analyses, Novagen discontinued making the assay we employed. However, for this study, we were not interested specifically in the absolute values of the samples, but rather comparisons between concentrations in segments and bags, and the

effect of storage for 24 hrs at 4C. Thus, while absolute values obtained using another assay might differ from those we obtained, our assessment of these comparisons, and of 24hrs of storage at 4C should remain valid. Further work is needed to confirm the reliability of this and other multiplexed diagnostic approaches including bead arrays, to study large numbers of leukocyte and platelet cytokines, in large numbers of samples from donor blood. If validated, the multiplex array technology offers the opportunity to expand our understanding of molecular and cellular changes in blood and blood products during storage. Its use can provide information on how concentrations and content of immunomodulatory, inflammatory, and vasoregulatory factors vary during storage, and how these changes correlate with transfusion reactions and effects on the recipient's pulmonary, endothelial, hematopoietic and other cells.

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FIG 1. IL-7 and IL-8 concentrations do not differ between segments (open bars) and bags (bars with diagonals) of 10-42 day old pre-storage leukoreduced PRBCs. Data are mean, 95% confidence interval. N=10 for each cytokine.

Fig 2.

Cytokine concentrations do not differ between segments (open bars) and bags (bars with diagonals) of 39-42 day old, non-leukoreduced whole blood. Data are mean, 95% confidence interval. N=10 for each cytokine except IL-1a (insufficient number of samples with measurable cytokines - no data presented)

Fig 3. Cytokine concentrations do not differ between segments (open bars) and bags (bars with diagonals) of 4-9 day old pre-storage leukoreduced platelets. Samples are those at time = 0 (not stored at 4C to test for effect of time). Data are mean, 95% confidence interval. N=10 for each cytokine

Fig. 4.

Linear regression (left) and Bland-Altman (right) plots of segment vs. bag cytokine concentrations in all erythrocyte preparations. Linear regression $r^2 = 0.95$ with a slope of 1.01 that did not differ from 1.00. Bland-Altman plot of difference (bag-segment) vs. mean of bag and segment concentrations with a linear regression (broken line) slope of 0.04 (not different from 0, $P=0.69$). $N=125$

Fig. 5. Linear regression (left) and Bland-Altman (right) plots of segment vs. bag cytokine concentrations in platelet preparations. Linear regression $r^2 = 0.97$ with a slope of 0.98 that did not differ from 1.00. Bland-Altman plot of difference (bag-segment) vs. mean of bag and segment concentrations with a linear regression (broken line) slope of 0.001 (not different from 0, $p=0.89$). $N=601$