Original Article

Release of cytokines in stored whole blood and red cell concentrate: Effect of leukoreduction

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Abstract:

Background: Storage time of blood components plays a major role in the accumulation of cytokines causing adverse transfusion reactions. **Aims:** The aim was to study the trend in the levels of interleukin-6 (IL-6), IL-8, tumor necrosis factor-alpha (TNF-α) and regulated upon activation, normal T-cells expressed and secreted (RANTES) during storage of whole blood (WB) and red cell concentrate (RCC) and to study the effect of leukoreduction (LR). **Materials and Methods:** WB sample was taken on 0, 7, 14, 21, and between 28 and 35 days and plasma aliquots were frozen. Samples from RCC and buffy-coat depleted RCC prepared using Optipress II were collected on 0, 7, 14, 21 and between 28 and 35 days. Cytokine estimation was done using ELISA development kits. Normal range of cytokines was established using 0 day samples of WB. Statistical analysis was done using nonparametric tests. **Results and Conclusion:** The normal range of IL-6 was 0-23 pg/ml, IL-8 0-12 pg/ml, TNF-α 0-3 pg/ml, and RANTES 1200-2000 pg/ml. IL-6 was in normal range and showed a decreasing trend during storage. IL-8 levels increased significantly from 0 to 35 days. In RCC, the highest level was 480 pg/ml on 28th day. It was in the normal range in buffy-coat depleted RCC up to 28 days. RANTES level was significantly low in buffy-coat depleted RCC compared to RCC. We conclude that WB has high levels of IL-8 and RANTES. The levels of cytokines are affected by storage period and LR. Comparison of WB and buffy-coat depleted RCC shows significantly low levels of IL-6, IL-8, and RANTES in buffy-coat depleted RCC. This study emphasizes the use of red cell components instead of WB and buffy-coat depleted RCC instead of RCC.

Key words:

Cytokines, interleukin, red cell concentrate, regulated upon activation, normal T-cells expressed and secreted, tumor necrosis factor, whole blood

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Introduction

In India, whole blood (WB) is still widely being used for transfusion instead of blood components. During storage period, many bioactive substances like cytokines are released.[1-3] Cytokines released by white blood cells (WBC) accumulate in the supernatant during storage of red cell concentrate (RCC). Leukoreduction (LR) is a potential means of preventing cytokine production. The aim of the present study was to estimate the levels of cytokines released during storage of WB and RCC. Important cytokines like interleukin-6 (IL-6), IL-8, tumor necrosis factor alpha (TNF-α) play a major role in febrile nonhemolytic transfusion reactions (FNHTR) and regulated upon activation, normal T-cells expressed and secreted (RANTES) is mainly involved in allergic reactions.[4-6]

Materials and Methods

Blood was collected from voluntary donors. Informed consent and their age, sex, address and parameters of medical examination were recorded in donor registration form. The donor was selected according to the donor selection criteria mandatory as per the Drug and Cosmetics Act, 1940 and rules 1945.[7] Blood was collected in different bags as per the requirement and stored at 2-6°C taking the day of collection as 0 day. Blood collected in a single bag was stored as WB, and the other bags were taken for component preparation.

Red cell concentrate was prepared by centrifuging WB at 5000 g for 5 min at 2-6°C and removing most of the plasma keeping hematocrit (Hct) 70% ± 5%.[8] For preparation of buffy-coat depleted RCC, 450 ml blood was collected in quadruple bags and was prepared using "top and bottom" method using Optipress II (Baxter, Fenwal) an automatic component extractor.[9]

For WB, RCC and buffy-coat depleted RCC 10 ml samples were taken from the bag aseptically in laminar air flow. One hundred and eighty samples of WB (30 samples each on 0, 7, 14, 21, and between 28 and 35 days) and 150 samples of RCC and buffy-coat depleted RCC were collected similarly. Hemogram was determined using hematology analyzer (Nihon Kohden, Japan). For buffy-coat depleted product, the WBC count was done using Nageotte chamber (Hausser No. 4000, Electron Microscopy Sciences, Hatfield, PA, USA). The sample was then centrifuged at 3500 rpm for 15 min at 2-6°C and the supernatant was separated and stored in aliquots at –56°C deep freezer for cytokine estimation on different days.

Cytokine assays

Cytokine assays were done using ELISA development kits which included a set of antibodies, standards, conjugate, and substrates. Uncoated ELISA plates were purchased separately, and ELISA for each cytokine was developed. Monoclonal antibody was coated on NUNC Maxisorp microwell plates overnight as per manufacturer instructions of reagents used. Each test included different dilutions of standards for the preparation of standard curves. On immobilized capture antibody detection, antibody was added followed by the addition of an enzyme and substrate producing a color product in proportion to the concentration of cytokine. The absorbance was read using ELISA reader at 450 and 570 nm for IL-6, IL-8, and TNF-α and at 405 and 650 nm for RANTES. The set of reagents used was of:

- eBioscience (USA) for human IL-6.
- BioLegend (San Diego, CA) for human IL-8 and TNF-α.
- PeproTech (NJ, USA) for Human RANTES.

The sensitivity of different cytokine assays was IL-6 = 2 pg/ml; IL-8 = 8 pg/ml; TNF-α = 2 pg/ml; and RANTES = 16 pg/ml.

For statistical analysis *t*-test, nonparametric Mann-Whitney *U*-test for inter-group and Wilcoxon signed rank test for intra-group comparison was used. $P < 0.05$ was considered significant.

Results

The age group of the donors was 29 ± 11 years. Among 30 donors, 23 were male and seven female. The hemoglobin of all donors was above 12.5 g/dl. RCC was prepared from 30 donors, 29 male, and 1 female. The age group of donors was 30 ± 6 years. The Hct was in the range of 65-75%. Buffy-coat depleted RCC was prepared from 30 donors, 28 male and 2 female with the age group 26 ± 6 years. The mean WBC count and platelet count in WB was $3.1 \pm 3.1 \times 10^9$ WBCs/unit and $241 \pm 96 \times 10^3$ platelets/μl, in RCC it was $3.68 \pm 1.1 \times 10^9$ WBCs/unit and $265 \pm 107 \times 10^3$ platelets/μl and in buffy-coat depleted RCC it was $5.5 \pm 1.6 \times 10^8$ WBCs/unit and $0.78 \pm 1.8 \times 10^3$ platelets/μl respectively. The comparison of mean values by *t*-test showed significantly low WBC count in buffy-coat depleted RCC ($P = 0.0$) compared to RCC and WB ($P = 0.00003$). Similarly, the platelet count of buffy-coat depleted RCC was significantly low compared to WB and RCC ($P = 0.0$).

The normal range of IL-6 was 0-23 pg/ml; IL-8, 0-12 pg/ml; TNF-α, 0-3 pg/ml and RANTES, 1200-2000 pg/ml.

The observed range of IL-6 in WB on 0 day was from 2 to 16 pg/ml. A trend of decrease in IL-6 levels was observed from 0 day to 28-35 days in WB, RCC, and buffy-coat depleted RCC Table 1. In buffy-coat depleted RCC, IL-6 levels were low up to 28 days and in the normal range. For comparison, absolute value of cytokine in each unit was calculated using the volume of plasma and the median and range is shown in Table 1.

Interleukin-8 was in the normal range on 0 day in WB. On 7th day, the level was low but increased on 14th day, and there was a steady increase continuously from 14 to 35 days. On 28 days, the observed range was 27-402 pg/ml. IL-8 increased progressively during storage in supernatants of RCC units also. On 0 day, the levels were in the range of 2-86 pg/ml increasing up to 89-80 pg/ml on 28 days. In buffy-coat depleted RCC, IL-8 was in the normal range throughout the storage period. The median and range of absolute levels of IL-8 are shown in Table 2.

Tumor necrosis factor-alpha level increased from the 7th day in few WB samples. On 35th day in two samples, the level reached 181 pg/ml. TNF-α level was in low range in RCC. In RCC, the level was up to 45 pg/ml on 28 days and in buffy-coat depleted RCC the level never exceeded 21 pg/ml. Table 3 shows the absolute median values and range of TNF-α.

Table 1: Comparison of absolute median IL-6 levels in 30 WB, RCC, and buffy-coat depleted RCC

Number of days	Median IL-6 pg/unit (range)		
	WB	RCC	Buffy-coat depleted RCC
0	817* (24-16,277)	365*† (8-13,182)	152* (30-1382)
7	499 (83-9682)	218 (59-5733)	145 (13-1386)
14	483 (56-5712)	209 (14-5540)	134 (9-1299)
21	333 (50-6525)	139 (19-1116)	132 (10-824)
Between 28-35	286 (46-2205)	104† (11-1001)	70 (8.7-555)

*Statistically significant decrease in IL-6 from day 0 over 28-35 days of storage in WB ($P = 0.0004$), RCC ($P = 0.011$) and buffy coat depleted RCC ($P = 0.036$); †Significant decrease in IL-6 on 0 day ($P = 0.007$) and 28 days ($P = 0.00$) in RCC compared to WB; WB: Whole blood; RCC: Red cell concentrate; IL-6: Interleukin-6

Table 2: Comparison of absolute median IL-8 levels in 30 WB, RCC, and buffy-coat depleted RCC

Number of days	Mediana IL-8 pg/unit (range)		
	WB	RCC	Buffy-coat depleted RCC
0	1502† (105-5423)	613†‡ (170-8358)	554†§ (161-1885)
7	4112* (340-10,931)	3069* (177-7820)	738 (208-2174)
14	13,797 (4519-40,513)	7067 (589-25,950)	813 (252-1431)
21	14,545 (4268-67,367)	11904 (1471-38,663)	955 (634-2602)
Between 28 and 35	27,455 (8270-90,470)	13547‡ (5188-61,922)	1124§ (472-2985)

*Statistically significant increase in IL-8 from day 0 to 7 days in WB ($P = 0.00$) and RCC ($P = 0.002$). †Statistically significant increase in IL-8 from day 0 over 28-35 days of storage in WB, RCC, and buffy coat depleted RCC ($P = 0.00$); ‡Significant decrease in IL-8 on 0 day ($P = 0.01$) and 28 days ($P = 0.00$) in RCC compared to WB; §Significant decrease in IL-8 on 0 day ($P = 0.03$) and 28 days ($P = 0.00$) in buffy coat depleted RCC compared to RCC. WB: Whole blood; RCC: Red cell concentrate; IL-8: Interleukin-8

Table 3: Comparison of absolute median TNF-α level in 30 WB, RCC, and buffy-coat depleted RCC

Number of days	Median TNF-α pg/unit (range)		
	WB	RCC	Buffy-coat depleted RCC
0	317*† (48-2337)	221*† (9-990)	34*† (9.3-1079)
7	348 (20-18,142)	227 (31-1212)	53 (9.6-1138)
14	499 (31-21,024)	246 (36-2862)	67 (6.4-1652)
21	524 (52-22,310)	280 (66-2455)	74 (9-965)
Between 28 and 35	556 (52-46,676)	300 (75-4133)	615 (8-2012)

*Statistically significant increase in TNF-α from day 0 over 28-35 days of storage in WB ($P = 0.009$), RCC ($P = 0.03$) and buffy coat depleted RCC ($P = 0.002$); †No significant difference in TNF-α level in WB, RCC, and buffy coat depleted RCC on 0 day and 28 days. WB: Whole blood; RCC: Red cell concentrate; TNF-α: Tumor necrosis factor-alpha

Table 4: Comparison of absolute median RANTES levels in 30 WB, RCC, and buffy-coat depleted RCC

Number of days	Median RANTES pg/unit (range)		
	WB	RCC	Buffy-coat depleted RCC
0	453,059 (171,771-710,850)	178,345*† (48,468-446,400)	1424*‡ (221-49,930)
7	538,276 (146,769-642,600)	184,402 (53,700-680,000)	1614 (447-49,308)
14	509,800 (250,128-610,663)	176,812 (107,900-406,528)	3034 (533-65,081)
21	513,400 (327,380-620,400)	183,008 (100,440-383,880)	3058 (976-205,920)
Between 28-35	500,530 (299,200-601,510)	202,200† (106,530-444,770)	4387‡ (720-186,120)

*Statistically significant increase in RANTES from day 0 over 28-35 days of storage in RCC (P=0.019) and buffy coat depleted RCC (P = 0.000); †Significant decrease in RANTES on 0 day (P = 0.00) and 28 days (P = 0.00) in RCC compared to WB; ‡Significant decrease in RANTES on 0 day (P = 0.00) and 28 days (P = 0.00) in buffy coat depleted RCC compared to RCC; RANTES: Regulated upon activation, normal T-cells expressed and secreted; WB: Whole blood; RCC: Red cell concentrate

Regulated upon activation, normal T-cells expressed and secreted level was very high from 0 day in the range of 856-2000 pg/ml which increased up to 35 days in the range of 1240-2000 pg/ml. The detection range of the kit was up to 2000 pg/ml hence >2000 was assumed as high levels, and the samples were not repeated in further dilutions. RANTES showed mean values of 57.58 ± 129.17 pg/ml in buffy-coat depleted RCC, which is much lower than that in RCC 1754.11 ± 409.98 pg/ml on 0 day. The levels in both the types of RCC increased from 0 day to 28 days. Absolute median values of RANTES are shown in Table 4.

Discussion

As cytokines are not included in a routine investigation like other hematological parameters, normal range of cytokines is not published. The manufacturers of the kits too give the expected values as per their samples tested. May be the importance of cytokines came in light later or may be limited studies are carried out due to the immense cost of measuring cytokines. Comparing the levels of cytokines in different components was not possible without normal range. This study established the normal range of IL-6, IL-8, TNF-α, and RANTES in WB of healthy donors. This first step was necessary to compare cytokine levels in RCC and LR-RCC, to study the time-dependent storage effect.

Interleukin-6 showed a decreased trend from 0 day to 35 days in WB. Similar results were reported by Jacobi *et al.* who found IL-6 levels in physiological limits in WB.[10] A study by Nielsen *et al.* reported that IL-6 was under the detection limit of 3.9 pg/ml in WB and levels remained low during the storage.[3] Thus, these findings are comparable with the present study concluding that IL-6 is not accumulated in WB stored at 4°C.

Mean leukocyte count 3.1 ± 3.1 × 10^9 WBCs/unit and platelet count 241 ± 96 × 10^3/μL in WB indicate that higher levels of leukocytes and platelets also contribute to increase in cytokine levels. IL-8 level increased on 14th day, and there was steady increase up to 35 days. TNF-α level increased gradually in WB after 14 days of storage. In 43% cases, the level remained 2-50 pg/ml up to 35 days of storage. The level of RANTES was also very high in WB and increased significantly during storage and may be attributed to platelets found. Comparing the absolute values, the median levels of all cytokines are more in WB compared to RCC and buffy-coat depleted RCC. This study conclusively suggests that WB has high levels of IL-8 and RANTES.

Davenport *et al.* quoted that many factors activate WBCs to generate cytokines during storage like activated complement components, thrombin or by cytokines released from damaged WBC or by nonbiological surfaces of plastic containers. The measure of sCD40L, a pro-inflammatory mediator released by leukocytes and platelet upon activation, indicates that WBC is activated, and cytokines are synthesized due to which the levels increase.[11] This study proves that cytokine concentration is directly related to WBC content and storage time.

It has been estimated that a freshly collected, WB unit contains 10^9 leukocytes, and their concentration continues to decrease with subsequent component processing.[12] There is a great variability in number of leucocytes in the components. The enumeration of residual leukocytes is important where traditional automated cell counters do not give accurate results at <100 WBCs/μl. Several methods are available, but Nageotte chamber has been found to be practical and cost effective in a blood center setting whereas other techniques like flow cytometry, cytospin method etc., are expensive, cumbersome, and labor intensive.[13-15] The WBC count was significantly low (P = 0.000) in buffy-coat depleted RCC as the majority of WBC are removed during processing. Similar study reported WBC count 4760 ± 3870/μl in RCC, which is comparable to the present study.[4] As the WBC count is reduced in buffy-coat depleted RCC, the risk of human leukocyte antigen (HLA) sensitization is also reduced. As quoted by Turner leucodepletion of blood reduces primary immunization to HLA and reduces the exposure to foreign antigens which are highly immunogenic.[16] The rate of alloimmunization varies from 7% to 44% among recipients receiving LR products and 20% to 50% in patients transfused with non LR products.[17-19]

Since red cells are stored at 2-6°C the possibility that residual or passenger WBCs in RBC units could synthesize and secrete cytokines during storage. Like WB, IL-6 levels were in the normal range in RCC. There was no sample showing the value above 23 pg/ml in 21 days. This study supports the study of Stack *et al.* where IL-6 levels remained low at any storage time.[4] Low levels of cytokines may have additive or synergistic effect in conjunction with other cytokines.

In the present study, IL-8 levels increased progressively from 2 pg/ml on 0 day to 480 pg/ml up to 28 days. There was no sample in normal range 14th day onwards. Higher levels of IL-8 have been reported by Stack *et al.* in RCC than in buffy-coat depleted RCC.[4] IL-8 has a priming effect on WBCs that renders them more sensitive to the effects of other pyrogenic cytokines.[20] IL-8 stimulates the migration of WBCs to sites of inflammation, the activation and degranulation of neutrophils and the basophile release of histamine as investigated in many other studies.[21-23]

Interleukin-8 levels are low in buffy-coat depleted RCC. WBC reduction prevented the accumulation of IL-8 during storage up to 28 days with a range of 2-14 pg/ml. It may be due to accumulation in packed RBC's which is explained by Haspl *et al*. to be released from RBC receptors into the packed RBC supernatant.[24] Chudziak *et al*. demonstrated that during the time lag, until component prestorage depletion unfavorable amounts of inflammatory cytokines accumulate in plasma. IL-8 increased more than 20 fold within 24 h of blood collection. They demonstrated that leucodepletion should be completed within 10 h of collection.[25] Since it has been proved that WBCs are the main source of cytokines and a significant decrease in WBC in buffy-coat depleted RCC proves that cytokines are much lower in buffy-coat depleted RCC compared to RCC.

Tumor necrosis factor-alpha level was low in RCC up to 45 pg/ml and 21 pg/ml in buffy-coat depleted RCC. Mild increase may be due to the stress level in blood donors as demonstrated by Haspl *et al*. which increases the activity of blood donor's immune cells.[24] Samples were taken from donors who donate blood may be for the first time and facing some stress which can influence cytokine levels. TNF-α level is not much elevated in either WB or RCC on day 1.

Regulated upon activation, normal T-cells expressed and secreted levels were high in both types of red cells although less in buffy-coat depleted RCC compared to RCC. The low platelet count in buffy-coat depleted RCC may be attributed to low RANTES level in them as platelets contain considerable amounts of RANTES, which is stored in α granules of platelet.[26] Literature mainly demonstrates RANTES in platelet concentrate as it is platelet-derived cytokine, but this study shows that RANTES is also found in low levels in RCC. Low platelet count reduces the accumulation of platelet-derived cytokines and alloimmunization to platelets as HLA class I antigens are expressed on platelets also. Thus, use of buffy-coat depleted RCC may have low platelet count, low cytokines, and reduced risk of alloimmunization.

At least 3% of all transfusions result in either FNHTR or allergic reaction.[27] As IL-6 is an endogenous pyrogen,[10] the presence of low-level IL-6 in WB or RCC may not cause FNHTR.[28] Lin *et al*. and Klüter *et al*. have reported the implication of proinflammatory cytokines IL-8 and TNF-α in RCC causing FNHTR.[29,30] As IL-8 levels are high in packed RCC transfusion may increase the chances of causing FNHTR as observed by Lin *et al*. in FNHTR patients showing high levels. The patients without febrile reactions did not have significant elevations of IL-6 and IL-8. Prevention of FNHTR is important because its manifestation, fever is a feature that is shared by other more dangerous complications of blood transfusions such as acute red cell hemolysis, sepsis from a contaminated product or transfusion-related acute lung injury. TNF-α has direct pyrogenic activity and can mediate inflammatory reactions.[31] RANTES is mainly involved in allergic reactions mediated by immunoglobulin IgE. It induces secretion of histamine, serotonin, and platelet activating factor which are released into the plasma causing hypotension. Allergic reactions include hives, urticaria, pruritus, erythema, bronchospasm, and hypotension which may be attributed to RANTES levels in the transfused blood component.[6]

Conclusion

Stored WB and RCC may release white cell and platelet-derived cytokines in a time-dependent manner and may be associated with many transfusion reactions. Comparative levels of cytokines show an increase in IL-8, TNF-α, and RANTES in a time-dependent manner in WB than in buffy-coat depleted RCC suggesting the use of buffy-coat depleted RCC instead of WB. Using buffy-coat depleted RCC may prevent many adverse effects due to leukocytes and cytokines which are low in buffy-coat depleted RCC. Prestorage white cell reduction is recommended for all red cell components.

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