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WHITE PAPER

Enrichment of Lymphocytes from Apheresis Residues

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Abstract

Obtaining an adequate number of human white blood cells (WBCs) can be a challenge for groups interested in using these cells to support cellular therapy research and applications. Residual cells from apheresis sets may offer an alternative source of human WBCs for research. This cellular product, however, also contains a significant number of red blood cells (RBCs), which can interfere with available WBC purification/separation methods. In order to isolate lymphocytes from this cellular source, a two-step process was developed. This process uses Hetastarch (HES) sedimentation, a common technique in cord blood banking, to reduce the RBC load in plateletpheresis residue. Lymphocytes are then isolated from the RBC-depleted, WBC-enriched supernatant by counterflow centrifugal elutriation (CCE), using the Elutra system. In practice, the HES sedimentation process removed 97% of the RBCs from apheresis residues that contained between 1E09 and 4E09 WBCs. The lymphocyte enrichment process yielded 7E08 to 1E09 lymphocytes, with a mean purity of 96%. These results demonstrate the ability of the Elutra system to isolate lymphocytes from a relatively small number of WBCs and from a relatively easily obtainable source.

Introduction

Human WBCs for laboratory use are often obtained from leukapheresis products or whole blood, or eluted from WBC reduction filters. An alternative source is plateletpheresis cellular residue, the cells retained in the disposable tubing set after a plateletpheresis procedure. These cells are "fully viable and functional." according Dietz et al. in a recent publication in Transfusion.[1] One potential drawback of plateletpheresis residue is the high RBC content. If this is an issue, RBCs can be removed by lysing with ammonium chloride or separated by density centrifugation, with or without a sedimenting agent. In this study, RBCs were removed from plateletpheresis residue using a HES sedimentation technique. HES sedimentation is a closed-system approach that has been used for the RBC depletion of umbilical cord blood, which also has a high ratio of RBCs to WBCs.

Following HES sedimentation, target cell populations can be isolated from the RBC-depleted plateletpheresis residue using the Elutra system. This closed-system device separates cells on the basis of their sedimentation properties (size and density). An elutriation profile was developed for the Elutra system for isolating lymphocytes from a relatively small number of cells.

Materials and Method

- Elutra Cell Separation System, Version 1.1 (Terumo BCT, Inc. - Catalog No. 7180x)
- Sterile Tubing Welder (Terumo Corporation – Catalog No. SC-201A)
- Plasma Extractor (Fenwal Laboratories – Catalog No. 4R4414)
- Elutra System Disposable Tubing Set, 1 each (Terumo BCT, Inc. - Catalog No. 70800)
- Accessory Platelet Storage Bag, 1 each (Terumo BCT, Inc. - Catalog No. 70030)
- Transfer Pack, 600 mL, 1 each (Charter Medical Limited - Catalog No. T3106)
- Phosphate Buffered Saline, Ca++ and Mg++ Free, 3L (Baxter Healthcare Corporation – REF EDR9865)
- 0.9% Sodium Chloride Injection USP, 1,000 mL (B. Braun Medical, Inc. - Catalog No. L8000)
- HESpan, 6% Hetastarch in 0.9% Sodium Chloride Injection, 75 mL (B. Braun Medical, Inc. - Catalog No. L6511)
- Plateletpheresis Cellular Residue, 1 each

HES Sedimentation of RBCs

Disposable tubing sets from plateletpheresis procedures performed on normal donors were transported within four hours post-procedure. The cellular residue in each set was drained into a citrate vinyl bag for storage overnight at 5° C to 10° C. The following day, the cells were diluted with PBS to a volume of 225 mL, and then the cell suspension was sampled and weighed. This initial sample was labeled "pre." To this cell suspension was added 75 mL of 6% HESpan, producing a final HES concentration of 1.5%. A sterile tubing welder was then used to connect the citrate vinyl bag to a 600 mL transfer pack. The citrate vinyl bag was allowed to sit, with the ports facing upward, for 40 minutes at room temperature. The citrate vinyl bag was then placed in a plasma extractor, where the RBC-poor, WBC-enriched supernatant was allowed to flow from the citrate vinyl bag to the transfer pack. The transfer was terminated when RBCs were observed exiting the citrate vinyl bag. The bag containing the RBC sediment was sampled and then discarded, and the transfer pack containing the WBC-enriched supernatant was further processed as described below.

Isolation of Lymphocytes by Elutriation

For each procedure, the previously described supernatant was weighed and a sample analyzed using a COULTER® AcT diff™ Hematology Analyzer. The WBC and RBC concentrations obtained from the automated cell counter, and the product volume (after removal of the sample), was recorded on a case report form. The starting product data was entered into the Elutra system after the tubing set was primed with media. The media consisted of the following:

- Primary Media: Phosphate Buffered Saline
- Secondary Media: 0.9% Sodium Chloride Injection USP

All procedures were conducted using the Elutra system profile configuration settings listed below:

- Media flow ramp = 0.15 mL/min/sec
- Centrifuge ramp = 528 rpm/min
- Cell:media ratio during fraction 1 = 1:1.0

Fraction	Flow Rate (mL/min)	Centrifuge (rpm)	Volume (mL)
F1 = Load	25	2,400	825
F2	102	2,400	975
F3	103	2,400	975
F4 = Rotor Off	103	0	250

During each procedure, observations and fraction weights were recorded on a case report form. After each procedure, all fractions were sampled directly from the fraction collection bags. All samples, including the initial sample of the plateletpheresis residue (pre) and the sample of the RBC sediment were analyzed in triplicate by an automated cell counter (COULTER® AcT diff™ Hematology Analyzer), and by flow cytometry. The Terumo BCT-validated flow cytometry assays used were as follows:

- Terumo BCT WBC Phenotyping by Flow Cytometry: Differentiating Lymphocytes, Monocytes, and Granulocytes using CD45/CD14
- Terumo BCT WBC Phenotyping by Flow Cytometry: Differentiating Lymphocyte Subsets using CD3/CD16+56/CD45/CD19
- Residual RBC: Detection of Residual RBCs in Platelet or Plasma Products using Anti-glycophorin A

Results

Information on the plateletpheresis residues that were used to conduct the Elutra system lymphocyte enrichment performance runs is summarized in Table 1. The average ratio of RBCs to WBCs in these cellular products was 157:1, in a total fluid volume of approximately 225 mL. The WBCs (mean ± standard deviation) were 68 ± 5% lymphocytes, 18 ± 7% monocytes, and 14 ± 5% granulocytes, as determined by flow cytometry.

Table 1. *Analysis of Plateletpheresis Residue (N=5)*

Cell Type	Antibody	Cell Number		
		Mean	SD	Range
RBC	-----	3.33E11	4.09E10	2.85E11 – 3.88E11
WBC	-----	2.43E09	9.43E08	1.44E09 – 3.76E09
Lymphocyte	-----	1.65E09	6.50E08	9.72E08 – 2.38E09
B Cell	CD3–/CD19+	1.98E08	8.85E07	1.19E09 – 3.47E08
NK Cell	CD16+/CD56+	1.90E08	1.16E08	7.72E07 – 3.28E08
T Cell	CD3+	1.15E09	3.70E08	7.74E08 – 1.55E09
Monocyte	CD14+	4.63E08	3.38E08	1.77E08 – 1.02E09
Granulocyte	-----	3.16E08	5.56E07	2.40E08 – 3.81E08

HES Sedimentation of RBCs

The HES sedimentation process reduced the ratio of RBCs to WBCs from 157:1 to 5:1, with an average WBC recovery of 90 ± 3%. This process was completed in 40 minutes.

Isolation of Lymphocytes by Elutriation

The lymphocyte enrichment results are summarized in Tables 2 and 3. These results indicate that 66% of the lymphocytes were recovered in fractions 2 and 3, with a lymphocyte purity of 96%. The results also indicate that although the lymphocytes were split almost evenly between fractions 2 and 3, the majority of the RBCs (following HES sedimentation) were in fraction 2. The lymphocyte enrichment process was completed in 58 minutes.

Table 2. *Lymphocyte Recovery Results (N=5)*

	Cell Recovery (%)				Normalized Cell Recovery (%)			
	Lymphocytes	**B Cells**	**NK Cells**	**T Cells**	**Lymphocytes**	**B Cells**	**NK Cells**	**T cells**
F2	33.0 ± 5.8	45.9 ± 12.6	32.3 ± 19.1	32.0 ± 7.6	38.4 ± 4.8	65.5 ± 8.4	34.8 ± 15.4	35.5 ± 6.8
F3	33.0 ± 5.6	10.4 ± 6.1	37.3 ± 7.9	38.1 ± 7.1	38.8 ± 6.9	14.3 ± 5.6	44.3 ± 14.8	42.5 ± 7.6
F2+F3	65.9 ± 5.2	56.3 ± 15.4	69.7 ± 13.7	70.0 ± 6.8	77.2 ± 3.7	79.8 ± 5.1	79.1 ± 6.8	78.1 ± 5.2

Data expressed as mean and (standard deviation), where cell recovery is a percentage of the starting cell number and normalized recovery is a percentage of the total cell number recovered from the HES sedimentation and Elutra system procedure.

Table 3. *Lymphocyte Purity Results (N+%)*

	RBC:WBC Ratio	WBC Purity (%)			Lymphocyte Purity (%)		
		Lymphocytes	**Monocytes**	**Granulocytes**	**B Cells**	**NK Cells**	**T Cells**
F2	4.7 ± 2.9	99.6 ± 0.5	0.3 ± 0.5	0.1 ± 0.2	17.8 ± 6.9	10.9 ± 7.0	70.1 ± 8.9
F3	1.4 ± 1.2	92.1 ± 6.5	7.7 ± 6.4	0.2 ± 0.1	3.7 ± 1.8	12.8 ± 6.4	82.6 ± 5.9
F2+F3	2.8 ± 1.8	95.9 ± 3.1	4.0 ± 3.1	0.1 ± 0.1	10.7 ± 4.1	12.2 ± 6.2	77.1 ± 7.2

Data expressed as mean and (standard deviation).

Discussion

The described protocol uses the Elutra system to isolate lymphocytes from plateletpheresis cellular residue, after RBC depletion of the residue by HES sedimentation. HES is an injectable colloid solution that induces RBCs to rouleaux and sediment. It has been used by cord blood banks for volume reduction and cryopreservation, and can reportedly bring about a mean RBC removal of close to 80% with a WBC recovery greater than 70%.[2] The HES sedimentation technique used in this study with plateletpheresis residue, is a modification of one described by Rubinstein et al.[3] It achieved a mean RBC removal greater than 95% with a WBC recovery of 90%, and generated a WBC-rich supernatant suitable for subsequent processing on Elutra system.

The lymphocyte enrichment process separates the cells in the WBC-rich supernatant by counterflow centrifugal elutriation. It uses PBS as the elutriation medium to elute lymphocytes in two separate fractions. The first fraction contains the majority of the B cells and residual RBCs, and the second predominantly T cells. After eluting the lymphocytes, the remaining monocytes and granulocytes are recovered in the rotor off fraction. This elutriation process can be done successfully with a relatively low number of WBCs, provided there is a sufficient number of monocytes plus granulocytes to maintain a stable cell bed while the lymphocytes are being eluted. The range in WBC number used in this study, prior to HES sedimentation, was 1.4E09 to 3.8E09, and the range in the number of monocytes plus granulocytes was 0.5E09 to 1.4E09.

Conclusion

A two-step protocol is reported for the isolation of lymphocytes by HES sedimentation of plateletpheresis residue, followed by counterflow centrifugal elutriation. This closed system approach yields lymphocytes with greater than 95% purity and greater than 65% recovery from initial starting cell products containing 1.4E09 to 3.8E09 WBCs.

Addendum 1: Cell Data (N=5)

Cell Type	Product		Fraction 1		Fraction 2		Fraction 3		Fraction 4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
***Cell Number*:**										
RBC	**3.33E+11**	4.09E+10	**2.38E+08**	1.17E+08	**1.98E+09**	8.24E+08	**6.65E+08**	5.10E+08	**9.45E+07**	6.02E+07
B-Cell	**3.05E+08**	1.19E+08	**1.31E+07**	5.82E+06	**9.75E+07**	6.98E+07	**1.99E+07**	1.33E+07	**6.84E+06**	4.56E+06
NK-Cell	**2.90E+08**	1.60E+08	**2.28E+06**	8.89E+05	**7.09E+07**	6.19E+07	**6.91E+07**	4.49E+07	-----	-----
T-Cell	**1.81E+09**	7.37E+08	**2.45E+07**	1.39E+07	**3.82E+08**	2.04E+08	**4.24E+08**	1.11E+08	**6.06E+07**	3.11E+07
Lymphocyte	**1.65E+09**	6.50E+08	**4.19E+07**	2.02E+07	**5.70E+08**	3.16E+08	**5.24E+08**	1.62E+08	**1.17E+08**	6.72E+07
Monocyte	**4.63E+08**	3.38E+08	**2.51E+06**	3.03E+06	**2.55E+06**	4.94E+06	**4.72E+07**	4.94E+07	**3.19E+08**	2.43E+08
Granulocyte	**3.16E+08**	5.56E+07	**2.02E+06**	2.27E+06	**5.27E+05**	5.52E+05	**9.99E+05**	1.05E+06	**2.25E+08**	6.96E+07
***Cell Recovery (%)*:**										
RBC	-----	-----	**0.1**	0.0	**0.6**	0.3	**0.2**	0.2	**0.0**	0.0
B-Cell	-----	-----	**7.7**	5.1	**45.9**	12.6	**10.4**	6.1	**3.4**	1.3
NK-Cell	-----	-----	**1.7**	1.6	**32.3**	19.1	**37.3**	7.9	-----	-----
T-Cell	-----	-----	**2.6**	2.1	**32.0**	7.6	**38.1**	7.1	**5.2**	2.4
Lymphocyte	-----	-----	**3.2**	2.5	**33.0**	5.8	**33.0**	5.6	**6.8**	2.3
Monocyte	-----	-----	**0.5**	0.5	**0.3**	0.5	**8.5**	4.3	**67.3**	9.2
Granulocyte	-----	-----	**0.7**	1.0	**0.2**	0.2	**0.3**	0.3	**70.5**	13.7
***Norm. Cell Recovery (%)*:**										
RBC	-----	-----	**0.1**	0.0	**0.6**	0.3	**0.2**	0.2	**0.0**	0.0
B-Cell	-----	-----	**10.8**	6.3	**65.5**	8.4	**14.3**	5.6	**4.8**	1.4
NK-Cell	-----	-----	**2.1**	1.8	**34.8**	15.4	**44.3**	14.8	-----	-----
T-Cell	-----	-----	**3.0**	2.4	**35.5**	6.8	**42.5**	7.6	**5.7**	2.3
Lymphocyte	-----	-----	**3.8**	3.0	**38.4**	4.8	**38.8**	6.9	**7.9**	2.2
Monocyte	-----	-----	**0.5**	0.5	**0.3**	0.5	**9.7**	4.6	**78.4**	7.7
Granulocyte	-----	-----	**0.9**	1.1	**0.2**	0.3	**0.4**	0.4	**86.6**	5.0
***Lymphocyte Purity (%)*:**										
B-Cell	**12.8**	3.1	**33.4**	12.1	**17.8**	6.9	**3.7**	1.8	-----	-----
NK-Cell	**11.6**	5.0	**6.8**	4.2	**10.9**	7.0	**12.8**	6.4	-----	-----
T-Cell	**74.6**	4.7	**58.3**	13.1	**70.1**	8.9	**82.6**	5.9	-----	-----
***WBC Purity (%)*:**										
Lymphocyte	**67.8**	5.4	**88.3**	14.9	**99.6**	0.5	**92.1**	6.5	**17.6**	6.9
Monocyte	**17.9**	7.2	**6.7**	8.4	**0.3**	0.5	**7.7**	6.4	**44.1**	16.3
Granulocyte	**14.3**	4.8	**5.0**	6.6	**0.1**	0.2	**0.2**	0.1	**38.3**	15.6

Data expressed as mean and (standard deviation).
RBC (in Product) Assay: COULTER® AcT diff™ Hematology Analyzer.
RBC (in Fractions) Assay: Residual RBC (Detection of Residual Red Blood Cells in Platelet or Plasma Products using Anti-glycophorin A).
B-Cell, NK-Cell, and T-Cell Assay: Flow Cytometry - (Differentiating Lymphocyte Subsets using CD3/CD16+56/CD45/CD19).
Lymphocyte, Monocyte, and Granulocyte Assay: Flow Cytometry - (Differentiating Lymphocytes, Monocytes, and Granulocytes using CD45/CD14).

References

1. Dietz AB, et al. "A Novel Source of Viable Peripheral Blood Mononuclear Cells from Leukoreduction System Chambers." *Transfusion* 2006; 46: 2083-2089.
2. Zingsem J, et al. "Cord Blood Processing with an Automated and Functionally Closed System." *Transfusion* 2003; 43: 806-813.
3. Rubinstein P, et al. "Processing and Cryopreservation of Placental/Umbilical Cord Blood for Unrelated Bone Marrow Reconstitution." *Proc Natl Acad Sci* 1995; 92: 10119-10122.

Notes to U.S. and Canadian Customers

1. This research method has undergone limited testing and is provided for information only. There are no data available to support the application of this method in clinical procedures. Operators using this method do so at their own risk and should refer to the cautionary remarks in the *Elutra Cell Separation System Operator's Manual.* Failure to correctly follow the procedure described may lead to reduced product recovery and purity of monocytes, or total loss of target cells.
2. Terumo BCT considers the Elutra system a processing device for laboratory use and not subject to pre-market clearance or approval by the U.S. Food & Drug Administration and Health Canada.
3. Cells collected or isolated using this device that are further processed to produce a therapeutic product(s) or vaccine(s) for clinical use may require advance regulatory clearance or approval. Compliance with all relevant country-specific regulatory requirements is the sole responsibility of the user.

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