A Modified Counting Method for Very Small Number of Residual White Blood Cells in Platelet Concentrates by Flow Cytometry

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フローサイトメトリーによる血小板製剤に残存する 微量白血球測定法の改良

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濃厚血小板製剤 (PC) 中の微量白血球混入量をフローサイトメトリー (FCM) で測定する方法はすでに多く報告されている。我々は、非標識のニワトリ血球 (cRBC) を内部標準として試料と同時に PI 染色し、Cytoron (オーソ・クリニカル・ダイアグノスティクス K.K.) を使用して細胞数を測定する方法を開発した。この方法で測定できる細胞数は 1.6×10^2 - 10^5 個/ml であり、非常に再現性も良く、従来の方法に比べて操作が簡単で短時間で測定できる。

また、測定に必要な検体量は 0.2 ml と少なく、濃厚血小板製剤 (PC) に混入する微量白血球数を測定するために有効な方法であり、製品管理に役立つと考えられる。

Key Words: white blood cell depletion, reduced WBC in blood component, residual WBC counting.

Summary

A modified flow cytometric (FCM) method for counting very small numbers of residual white blood cells (WBCs) in platelet concentrates (PCs) was developed. Non-labeled chicken red blood cells (cRBCs) were added to samples of PCs as an internal control. After incubation with propidium iodide (PI) staining agent, residual WBCs and control cRBCs were counted using a Cytoron flow cytometer (Ortho-Clinical Diagnostics K.K. Tokyo, Japan). Average time needed to count one sample was 40 seconds. The range of cell numbers able to

counted by this method was 1.6×10^2 - 10^5 cells per ml with good reproducibility.

The advantages of this technique are that its rapid and accurate, requires only 0.2 ml of PCs and is easy to perform. It therefore represents a useful method for quality control of residual WBCs in platelet concentrates.

Introduction

White blood cell (WBC)-reduced PCs may diminish the risk of graft versus host disease (GVHD), sensitization to WBC antigens and viral transmission. As newer WBC filters are developed, methods for counting residual WBCs

in PCs become crucial. Various WBC counting techniques have been developed and published^{1)~5)}. Vachula et al⁵⁾ recently reported a method using flow cytometry (FCM), in which they added a known amount of fluorescein (FITC)-labeled chicken red blood cells (cRBCs) to samples of PCs, stained the mixture with PI reagent, and then counted the number of WBCs using a two-color flow cytometer.

Although this technique is accurate, it is not easy to perform, since the FITC-labeled cRBCs are unstable, and the intensity of FITC decreases over time. When this occurs, there is no clear separation between cRBCs and WBCs on FCM pattern.

We describe a modified technique using cRBCs not pre-labeled with FITC as the internal indicator. A known amount of unlabeled cRBCs were added to PCs samples, then stained

with PI reagent. All cells were counted using a single color detector by FCM and the distinction between WBCs and cRBCs was clear in terms of the peak channel of red fluorescence.

This method was easy to perform and proved to be useful for the routine quality control testing of PCs for residual WBCs.

Materials and Methods

Collection of PCs:

PCs from normal blood donors were prepared using Cobe Spectra (Cobe Lab. K.K.) or CS-3000 plus (Baxter Healthcare Corp. IL., USA) instruments.

Preparation of PI reagent:

PI reagent was prepared by mixing 1 ml of Triton-X 100, 100 mg of sodium citrate, 500 U of RNAase (Sigma Chemical Corp., USA) and 0.1 ml of ethanol containing 5 mg propidium iodide

Fig. 1 Procedure for preparing cRBCs for use as internal control for counting WBCs.

Fig. 2 Procedure for PI staining and FCM method. The cRBC reagent is adjusted to 3150 cells per ml.

Fig. 3 Wright's/ Giemsa staining of cRBCs (a) and human RBCs (b).

(Sigma) with 99 ml of distilled water.

Preparation of cRBCs:

cRBCs were purchased from Nippon Bio-Supp. Center K.K. and prepared as described in Fig. 1. In brief, the cRBCs were washed with phosphate buffered saline (PBS). Contaminating WBCs were removed by filtration (Pall PL50) and the cRBCs were washed again with

Alsever's solution. The cells were then centrifuged at 3,000 rpm for 5 minutes, the supernatant was discarded and the packed cRBCs were resuspended to 0.2% in Alsever's solution. This cRBC suspension was then diluted 1 in 2 (v/v) with 0.5% bovine serum albumin (BSA)-Alsever's solution. This sample could be stored for 3 months in good condition. If stored

Fig. 4 Cytograms of FITC-labeled cRBC and cRBC stained with PI reagent. Freshly prepared FITC-labeled cRBCs (a), freshly prepared cRBCs stained with PI reagent (b), and after 10 days storage (c, d).

more than 3 months, however, the cRBCs began to lyse. The cRBCs were adjusted to 4.0×10^6 per ml using a Nageotte hemocytometer. The cRBCs were stained with PI reagent and the flow rate of Cytoron was adjusted to 3,150 cells for 40 seconds' counting. This cRBC suspension was dispensed into tubes and stored at 4°C. PI-staining and FCM method:

Fig. 2 shows the protocol for testing a PC sample. 0.2 ml of PC sample and 0.025 ml of cRBCs were added to a tube and the tube was filled with PI reagent to a volume of 1 ml. The Cytoron (Orgho-Clinical Diagnostics K.K., Tokyo, Japan) was used for FCM counting, with calibration each day using control beads (Orgho-Count Calibration Kit) to verify the flow rate and calculate the reaction mixtures. The Cytoron was set so that measuring time using the control beads was 40 seconds (The best coefficient of variation value was 40 seconds). 0.025 ml of the unlabeled cRBCs suspension stained with PI reagent was adjusted to 3,150 cells for 40 seconds using the Cytoron. Counting with the Nageotte hemocytometer: Samples were diluted 1 in 5 (v/v) with Türk solution. 0.05 ml was loaded into the Nageotte chamber and the total number of WBCs was

Table 1 Reproducibility of test results using the Cytoron

No.	Number of WBCs in PC ($\times 10^3$ cells/ml)
6860	0.88 ± 0.35
6278	0.88 ± 0.35
6571	1.25 ± 0.46
1476	2.88 ± 0.35
1478	1.63 ± 0.52
3781	15.3 ± 0.89
7598	64.5 ± 0.93
2414	108.0 ± 1.16
2514	207.0 ± 7.29
Mean $\pm$ 1 S.D. (n=8)	

counted.

Results

Wright's/Giemsa staining of the nuclei:
Fig. 3 shows cRBCs stained with Wright's/Giemsa. Wright's-stained nuclei of cRBCs are illustrated.
FCM analysis of the cRBCs and contaminating WBCs:
Cytograms of FITC-labeled cRBCs⁵⁾ and PI-stained cRBCs are shown in Fig. 4. Fig. 4a shows an analysis of freshly prepared FITC-

Fig. 5 FCM cytogram of PI-stained cRBCs and human WBCs using freshly prepared cRBCs (a) and cRBCs stored for three months (b).

Fig. 6 Standard curve for counting dilutions of low WBC concentrations.

Fig. 7 Correlation between measurement of WBCs by Cytoron and the expected cell counts using a Nageotte hemocytometer.

labeled cRBCs while Fig. 4b shows freshly prepared cRBCs. Ten days later, the intensity of the FITC-labelled cRBC was decreased (Fig. 4c), but that of the cRBCs stained with PI was stable (Fig. 4d).

In contrast, Fig. 5 shows cytograms using our

modified method in which both cRBCs and contaminating WBCs were stained with PI reagent. Cytograms of freshly prepared cRBCs (a) and 3-month-old cRBCs (b) showed a clear separation between cRBCs and the contaminating WBCs. The cRBCs were stable for 3 months

Fig. 8 Cytogram and number of PI-stained cells in PCs prepared with a Cobe Spectra (1) and the CS3000 plus (2). The number of WBC nuclei (A) and that of cRBC (B) are counted. Thus, $\frac{\text{Number of B}}{\text{Number of A}} \times \left(\frac{4 \times 10^6 \times 0.025}{0.2} \right) \times 10^3 \text{ cells/ml}$ 0.025 ml of cRBCs ($4 \times 10^6 \text{ cells/ml}$) and 0.2 ml of sample were added to a total volume of 1 ml of reaction mixture.

Table 2 Measurement of WBCs (a) before filtration and (b) after filtration by the two methods

Experiment no.	Measurement ($\times 10^3 \text{ cells/ml}$)			
	Hemocytometer Cell number		Flowcytometer Cell number	
	(a)	(b)	(a)	(b)
6533	870 $\pm$ 30	N.D.	929 $\pm$ 10	0.2 $\pm$ 0.1
5755	210 $\pm$ 18	N.D.	225 $\pm$ 7	1.0 $\pm$ 0.2
5771	150 $\pm$ 12	N.D.	186 $\pm$ 8	0.2 $\pm$ 0.1
6175	150 $\pm$ 13	N.D.	160 $\pm$ 9	0.7 $\pm$ 0.2
4326	200 $\pm$ 17	N.D.	223 $\pm$ 10	0.5 $\pm$ 0.2

N.D.: not detected Mean $\pm$ 1 S.D.(n=4)

in 0.5% BSA-Elsever's and could be used as an indicator of control cells in this FCM method. Reproducibility of test and serial dilutions:

Reproducibility of test results by this method with the Cytoron are shown in Table 1. PI-stained WBCs in 0.2 ml samples of 9 PCs were counted 8 times using the Cytoron. The results revealed good reproducibility.

A representative standard curve is shown in

Fig. 6. A sample of 1×10^5 WBCs per ml counted by the hemocytometer method was used. Serial dilutions (1 in 10) of samples in PBS were prepared and counted by the Cytoron and Nageotte hemocytometer techniques in parallel. As shown in Fig. 6, the test results of the assay were linear when plotted on a logarithmic scale from 1,000 to 100,000 WBCs per ml. Correlation between the Cytoron and Nageotte hemocytometer methods:

Fig. 7 shows the correlation between cell numbers counted by the Cytoron and Nageotte hemocytometer methods in the range below 5×10^3 WBCs per ml. From the slope of the line obtained, measurements by the hemocytometer method were about 10-20% less than those by FCM.

Number of WBCs in PCs samples:

The cytograms and number of WBCs in PCs samples from normal donors prepared by the Cobe Spectra (1) and the CS-3000 plus (2) are shown in Fig. 8. The samples had very low levels of residual WBCs.

Before and after filtration:

Table 2 shows measurements of WBCs by the two methods both before (a) and after (b) filtration using a Pall PL50 filter. The FCM method detected all nucleated cells, whereas the Nageotte hemocytometer method was unable to detect all cells.

Discussion

As the technology for preparation of WBC-reduced PCs has advanced, a need has developed for a method to measure very small numbers of contaminating WBCs in these PCs.

The traditional hemocytometer method was improved by staining the WBCs with Türk solution. Recently, a number of authors have described another counting method using FCM. Dzik et al¹⁾ reported a method that analyzed the number of WBCs in samples from PCs using the combination of green and red fluorescence dyes on the FACS can (Becton-Dickinson, Mountsain View, CA, USA). Takahashi et al³⁾ obtained a sensitivity of 1.77×10^2 cells per ml without an internal indicator using the Cytoron Absolute (new model of Cytoron, Ortho-Clinical Diagnostics K.K. Tokyo, Japan). The Cytoron Absolute can define the number of cells in the flow cell. The older model Cytoron used in this study does not have this capacity. Instead, cRBCs were used as the internal indicator of the number of cells per flow cell.

Wenz et al⁴⁾ reported a sensitivity of 0.02 cells per μL . Their method required analysis of the total volume in the sample tubes, including the addition of extra buffer to flush the sidewalls of the tubes to ensure that all cells entered the flow stream. The average time needed was 20 minutes including staining with PI reagent and measurement using an Epics apparatus (Coulter Diagnostics). In addition, the risk of introducing air bubbles into the flow cells was great.

Vachula et al⁵⁾ reported a method using a known number of FITC-labeled cRBCs as an indicator of the number of cells analyzed and counted WBCs in samples from PCs using this

dual stain with the FACScan instrument.

We have modified their method to eliminate the FITC stain because it is unstable and decreases in intensity over time. We used PI reagent to stain the nucleus of the cRBCs. It was also found that the large nuclei of cRBCs are more sensitive to staining with PI reagent than FITC reagent (Fig. 3A).

The characteristics of the method described here are as follows: the number of contaminating WBCs ranged from 1.6×10^2 – 10^5 cells per ml; reproducibility test results were good; and the average time needed for counting was 40 seconds. Further, the method is based on a single stain using PI reagent for the nucleus of both human WBCs and cRBCs.

These results show that this modified method for counting residual WBCs in PCs is sensitive, accurate, rapid and simple for routine use.

Conclusion

This method counts residual WBCs in PC samples of 0.2 ml. The method is rapid and easy to perform.

We consider this method to be useful for the quality control of PCs in routine testing.

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