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Enumeration of residual cells in leucodepleted blood products: techniques and pitfalls

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Introduction

With the implementation of universal leucodepletion of blood products, quality assurance of cell depletion processes became a demanding issue that required both new quantification technologies of residual cells as well as statistical considerations in order to overcome hurdles inherent to the endeavour. Growing customer's expectations regarding standardised blood products and new techniques of leucodepletion, such as in-line filtration and apheresis technology, also require modern and quick validation tools. Finally, only flow-cytometry revealed the fact that even thawed fresh-frozen plasma (FFP) may contain enough viable lymphocytes to induce harm to the recipient [18], thus underlining the impact of modern quality control technology. In this article, we will give an overview of current technologies for quantifying residual cells in leucodepleted blood products and we will present some of own experience with such techniques. To finish, we will discuss theoretical considerations to be taken into account when working in this area.

Requirements of state-of-the-art technology for quality control

The internationally accepted quality standards of leucodepleted blood products are defined as 1 x 10^6 to 5 x 10^6 white blood cells (WBC) per unit, checked on 1% of units produced and 90% to 95% of investigated units fulfilling the requirements [3,4,26]. Depending on the numbers of blood products produced, these requirements create a heavy workload for the quality control laboratory and are demanding in terms of human, technical and financial resources. The ideal cell enumeration technique must fulfil a long list of clearly defined features, such as high reproducibility and accuracy, being an easy, straightforward procedure and allowing batch analysis of samples. Other important issues are unrestricted possibilities of data re-analysis and data storage as well as convincing data presentation and cost effectiveness are important. Most of the techniques currently available fulfil several of these requirements, but none of them fulfils them all [10].

Techniques for enumerating residual cells

Cell Coulter devices

Cell Coulter devices have been developed for clinical use and are clearly inadequate to be applied for quality control of blood products. The concentration of residual cells in blood products is 2-3 logs below the linearity range of such devices which are certified to investigate samples from patients with components at physiological concentrations. Since blood products are artificial solutions and suspensions, they are not suitable for investigation by Coulter devices manufactured for clinical purposes.

Microscopic counting techniques

Microscopic enumeration of blood cells by chamber counting is a traditional technique that was adapted in many ways for clinical and laboratory applications [8,10, 24, 25, 27, 30]. After sample dilution, optimal *in vitro* induced haemolysis and cell staining, the sedimented blood cells are counted microscopically in a defined volume, which is given by the various chambers available (Nageotte, Neubauer, Ballast). By knowing the investigated volume, the dilution factor and the number of events observed, the final concentration of contaminating cells in the product can be calculated. The chamber volume largely defines the theoretical sensitivity of the test. However, due to poorly standardised preparation steps, subjective data acquisition and inherent statistical errors as a consequence of quantification of low numbers of events, the methodological error is exceedingly high at the given thresholds. In addition, the methods are cumbersome, time-consuming, demanding on human resources and improbably comply with the requirements of statistical process control [34]. In the context of universal leucodepletion, chamber counting techniques can, therefore, no longer be considered as the gold standard and should be substituted by other, technically more sophisticated methods.

Molecular-based enumeration techniques

Genomic DNA amplification and its specific detection by hybridisation or sequencing techniques provide extraordinary sensitivity and specificity in investigating biological material. However, adapted protocols for quantification of residual blood cells in blood component products have major draw-backs. The sensitivity is no better than 1 cell/μL and the specificity is inferior to that of chamber techniques [21]. Furthermore, the multi-step laboratory work-up, including DNA extraction, amplification and detection as well as simultaneous analysis of limited-diluted control DNA as quantification standard, makes the method very time-consuming and difficult to apply.

Flow-cytometry

The combination of fluid mechanics (hydrodynamic focusing), optical signal generation by laser technology, digital signal transformation and computer analysis technology are the main principles of flow-cytometry (fluorescence activated cell sorting;

FACS) and provide a rapid and precise particle analysis of suspensions. By including microbeads into the system as an internal quantification standard, FACS has become a powerful, fast and accurate method for quantifying residual cells in blood products [11]. In addition the various high-quality monoclonal antibodies provided by the industry allow customisation of flow-cytometric protocols for specific needs. Moreover, the acquisition of electronic data provides optimal conditions for data re-analysis and long-term data storage. FACS is likely to become the new gold standard for cell quantification of blood products. Therefore, efforts to standardise protocols as well as interlaboratory know-how transfer will be important to foster this promising technology of quality control. However, the main hurdles of flow-cytometry are high costs and considerable technical expertise required to use this new technology properly.

Single platform FACS method

Since flow-cytometry only allows measurement of only relative numbers of cells, there have been various attempts to complete the system (dual platform measurement, volumetric triggering) [6, 12] in order to yield absolute cell numbers. However, only with the introduction of an internal quantification standard (single platform technique) [23, 30, 33] could the methodological errors be controlled and flow-cytometry became a widely accepted, reliable and quick method for particle quantification in blood products.

Quantification of white blood cells

Based on stable nuclear staining by propidium iodide, various kits and automated software packages for direct quantification of residual WBC (rWBC) are commercially available. The differences between the products are the accuracy of dispensing microbeads (quantification standard) to the sample and the gating options. In our laboratory, we introduced the LeucoCount® System from Becton Dickinson for rWBC quantification and adapted the system for counting residual red blood cells (rRBC) as well as residual platelets (rPLT) in plasma products (see below).

Table I shows the validation data examining the reproducibility and linearity of rWBC quantification by single-platform FACS analysis in heavily leucodepleted blood products. At the critical rWBC levels (< 5 rWBC/µL), standard products such as red cell concentrates (EC), platelet concentrates (PC) and fresh-frozen plasma (FFP) containing

Table I - Reproducibility and accuracy of residual cell enumeration in leucodepleted blood products by FACS

Product	Spike level * (cell/µL)					
	10	5	4	2	1	R^2
EC	13,2±0,83	6,72±0,44	5,83±0,13	2,27±0,35	1,02±0,44	0,9950
PC	13,7±1,53	6,94±0,33	5,49±0,53	2,54±0,08	1,36±0,19	0,9996
FFP	11,7±0,41	5,45±0,22	4,43±0,47	2,14±0,19	1,36±0,08	0,9970

* Each spike level was measured at least three times in indipendent sample preparations

a known number of rWBCs (spike), were repeatedly investigated. Briefly, the products were first leucodepleted by double filtration (EC and FFP) or by apheresis technology (PC) and then spiked by adding-back buffy-coat WBCs to a concentration level that could be determined by a Sysmex K-1000 device. The spiked products were then sequentially diluted with leucodepleted, not-spiked products as diluent to reach the spike levels of interest. At each spike level, three determinations of rWBCs by single-platform FACS (LeukoCount®, Becton-Dickinson) were performed. The data are given as mean ± standard deviation and linear regression (R^2) was applied to compare the mean of measured rWBC concentrations and the expected values. As shown by the data, in all products tested, FACS could reliably quantify the expected concentration of rWBCs over the entire range of critical rWBCs concentrations.

In a similar approach, we evaluated the reproducibility of rWBC and rRBC quantification over time (Figure 1). Investigating spiked ECs, we were able to show that rWBCs of critical concentration can be quantified accurately by FACS over more than 7 days if the EC is stored at +4° C. Similarly, rRBC quantification at all spike levels gave reliable results over prolonged periods of time (Figure 1/B). Together, these data suggest that single-platform FACS is most suitable for accurate quantification of rWBCs and rRBCs in leucodepleted blood products over extended periods of time, allowing the laboratory work-up to be economized by batch analysis, e.g. once a week.

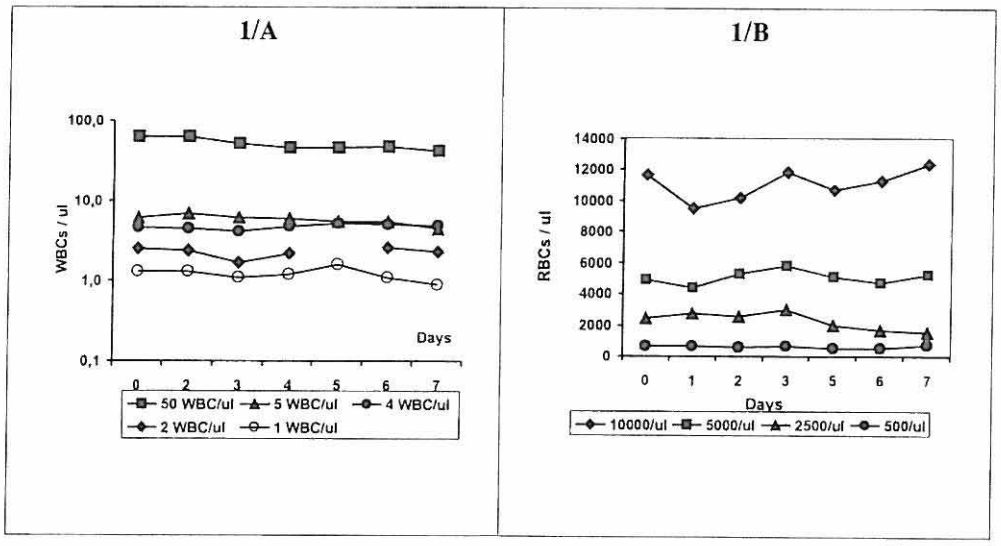

Figure 1 - *Reproducibility of residual cell enumeration in leucodepleted blood products over time.* ***Figure 1/A:*** *rWBC in EC.* ***Figure 1/B:*** *rRBC in PC.*
[Leucodepleted EC and PC were spiked with various concentrations of WBC and rRBC (for details see text). Over seven days, the residual cell concentrations in the products were measured by the FACS methods as described. The data show that, at the critical concentrations for rWBC (<10 rWBC/µL) and rRBC (< 6.000 rRBC/µL), the residual cells can reliably be enumerated over a prolonged period of time, provided the products are stored appropriately.]

Quantification of residual red blood cells and platelets

Modern product specifications of PC and FFP define restrictive levels of residual red blood cells (rRBC) and residual platelets (rPLT) in the respective products [4, 7, 17, 26]. As for quality control of contaminating rWBC, the determination and documentation of rRBCs and rPLTs in blood products have become a demanding task for the manufacturer of blood products. By adapting the single-platform FACS technology for rWBC quantification, we developed a FACS-based method for quantification of either rRBC or simultaneously rRBC and rPLT [13,15]. Instead of using nuclear staining by propidium iodide (suitable for rWBC), we introduced fluorochrome-conjugated monoclonal antibodies to stain specifically the red cell membrane epitope, glycophorin-A, and the platelet membrane epitope, glycoprotein IIb/IIIa (CD41a). However, in order to prevent cell aggregation, which would impair the quantification procedure, the antibodies were titered against the membrane epitopes. Given the exceedingly high copy number of glycophorin-A on the red cell membrane (>10^6 molecules per cell [16], anti-glycophorinA-PE (Pharmingen, San Diego) was prediluted (1:50-1:250) in buffer solution and stabilised by adding 0,5% bovine serum albumin. For rPLT quantification in FFP, it turned out that the rPLT concentration is low enough to obtain saturating staining of CD41a using undiluted anti-CD41a-FITC as provided by the manufacturer (Pharmingen, San Diego). Figure 2 shows the gating of the original LeucoCount® kit for quantification of rWBCs in EC, PC and FFP using nuclear staining by propidium iodide (Figure 2/A).

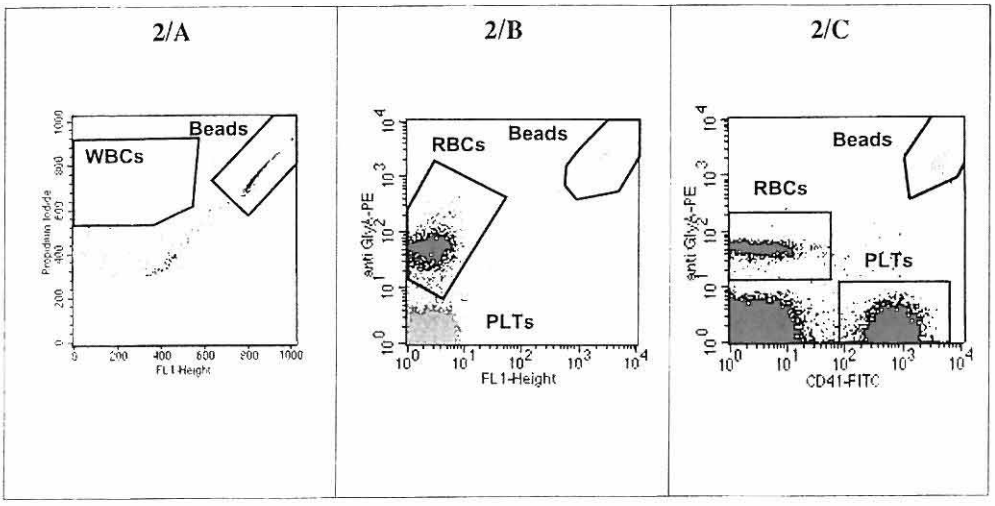

Figure 2 *- Multiparameter staining of residual cells in leucodepleted blood products.*
Figure 2/A: *rWBC in EC.* ***Figure 2/B:*** *rRBC in PC.* ***Figure 2/C:*** *rRBC and rPLTs in FFP.*
[Residual cells in various blood products were stained either by propidium iodide-staining for nuclei of rWBC (Figure 2/A) or a cell membrane anti-glycophorinA-PE for rRBC and anti-CD41a-FITC for rPLT (Figure 2/B and 2/C).
The target population is clearly discriminated from debris and microbeads, which allows it to be captured in the measurement gate.
The quantification of rRBCs and rPLTs in FFP is done in the same tube by simultaneous staining of RBC and PLT (Figure 2/C).]

The adapted kit using membrane staining with anti-glycophorinA-PE for rRBC quantification in PC and anti-glycophorinA-PE together with anti-CD41a-FITC to quantify rRBCs and rPLTs simultaneously in FFP is shown in Figure 2/B and 2/C.

Since the number of events acquired for quantification of rRBCs and rPLTs is about 100 - 1.000 times larger than the number of acquired events for rWBC quantification, the accuracy of rRBC and rPLT determination is higher that that of rWBC determination. In summary, single-platform FACS methods, using either surface staining of red cells and platelets or nuclear labelling of white blood cells, provide an elegant method for quantifying rRBCs, rPLTs and rWBCs, allowing batch analysis of samples.

Pitfalls in flow-cytometry

Although the analytical capacity of FACS is impressive, the technique can only produce meaningful data if some basic conditions are adhered to strictly.

First, it is important, to run unstained as well as stained controls with each batch of analysis to define the proper gating. We recommend that commercially available, fixed cells are not used as controls since their physical properties (size, shape, surface complexity) may be substantially different from the properties of fresh cells and therefore may be misleading in defining the acquisition gate in the FSC/SSC plot. To overcome this problem, we run a newly prepared spike control using fresh cells with each batch of analysis. The proper fluorescence compensation is also of some importance, especially when performing simultaneous staining for rRBC and rPLT. Here, the commercially available calibration beads are of paramount value in defining the accurate instrument settings.

Secondly, if surface staining by monoclonal antibodies is performed, the proper performance of the antibody must be checked by inclusion of a positive control sample. The antibody can lose its activity rapidly, particularly if it has been prediluted. An antibody dilution procedure that takes care of proper protein stabilisation and pH control must be applied. Furthermore, antibody titration must be checked as soon as cell aggregates are observed.

Thirdly, one must ensure that the acquisition gate contains the complete cell population of interest and, also, that the excluded events are well controlled (e.g. exclusion of debris only) in order to prevent unacceptable data loss. Recently, Janatpour *et al.* summarised the atypical patterns of flow-cytometric analysis files encountered on quantification of rWBC in red cell components [19], underlining the importance of proper gating of each single sample analyzed. Since the data can be stored as a list mode file, the supervisor can review the gating strategy as well as other morphological features of the cell populations independently of data acquisition. Therefore, we recommend visual double-checking of the proper gating by the supervisor before the data are validated.

Fourthly, the survival time of WBC subpopulations differ substantially in the various blood products [14]. Apoptotic WBC may cause an overestimation of rWBC by nucleus fragmentation. On the other hand, the physical features of disintegrated nuclei vary widely, so that nuclear fragments may fall outside the measuring gate, which will result in underestimation of rWBCs. Figure 3 shows the decay of WBC nuclei over time in buffy-coat-depleted EC and in the corresponding FFP. Polynuclear neutrophils, which are enriched in the rWBC population of EC, are particularly prone to undergo nuclear fragmentation over time (Figure 3/A). Since the rWBC population in the corresponding FFP is composed mainly of mononuclear cells, there is little nuclear fragmentation in

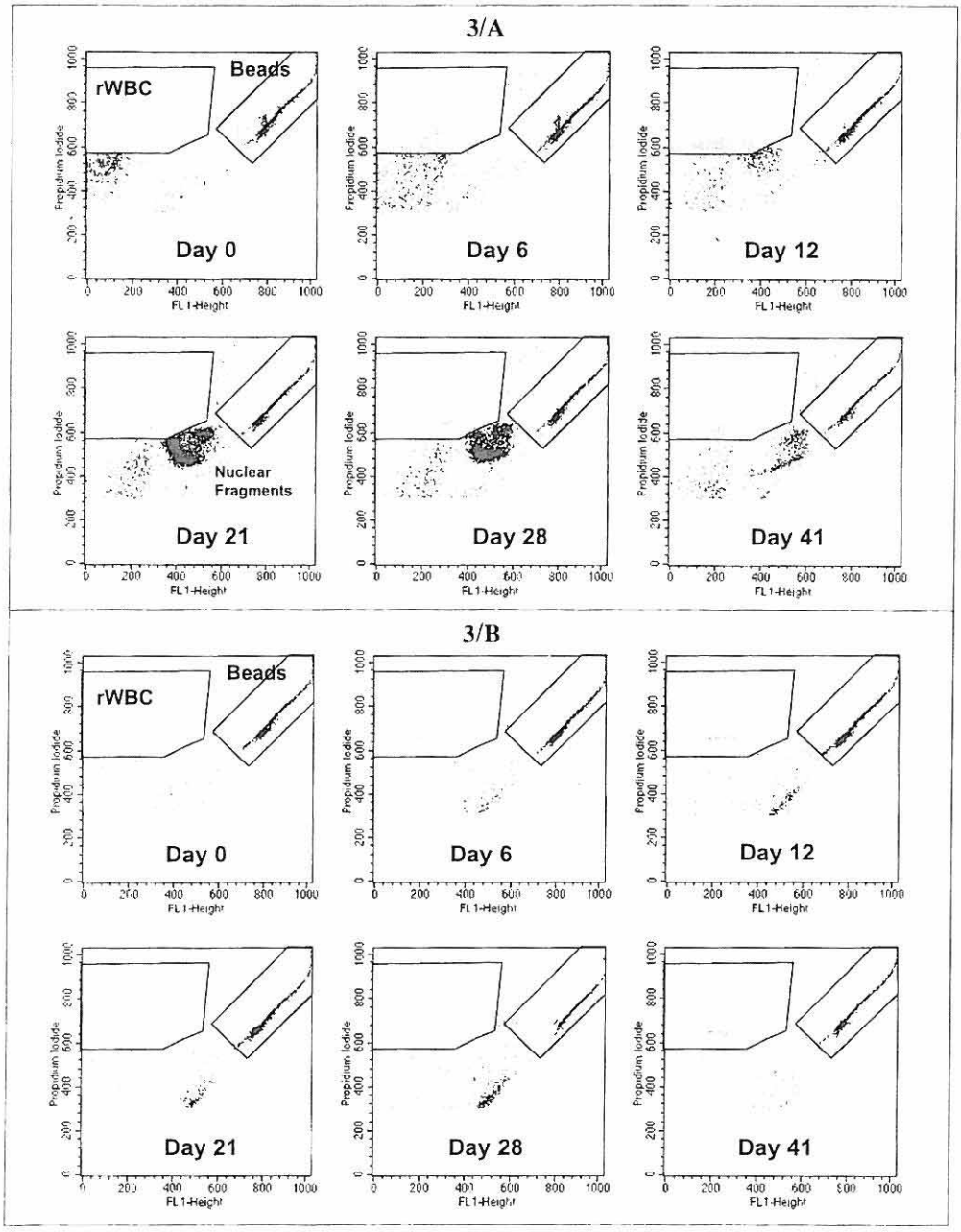

Figure 3 - ***Figure 3/A:*** *nuclear staining of rWBC in EC.*
Figure 3/B: *nuclear staining of rWBC in FFP.*
[Time course staining of nuclei of rWBCs by propidium iodide in buffy-coat-depleted EC and FFP reveals time-dependent decay of some of the nuclei, occurring mainly in the EC. As shown in Figure 3/A, at day 0 the rWBC population in EC is composed of two different populations, one of which undergoes fragmentation over time. The FFP contains many fewer fragmented nuclei (Figure 3/B).]

FFP over time (Figure 3/B). As shown in Figure 4, mononuclear and polynuclear populations can easily be discriminated by data analysis of the FSC/FL1 plot.

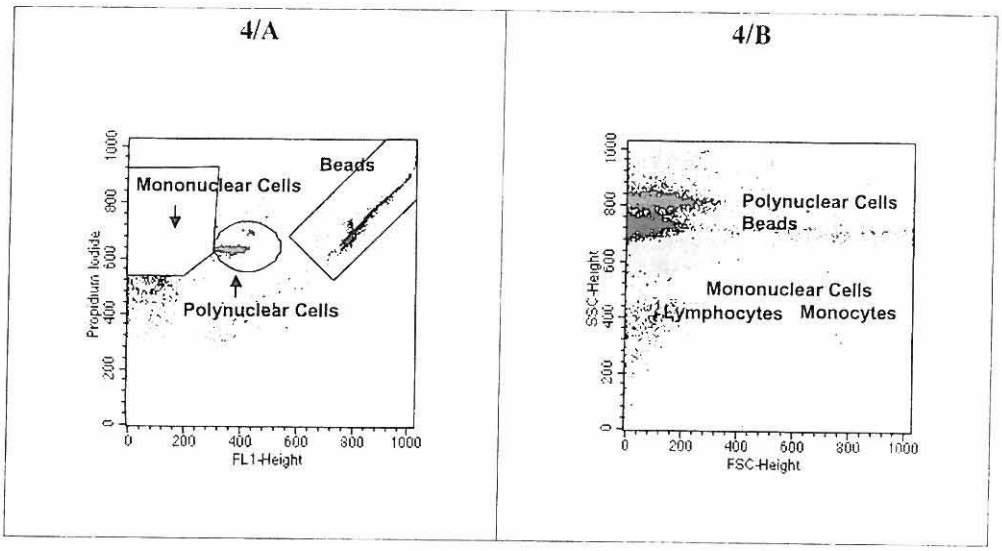

Figure 4 - *Nuclear analysis of rWBC in buffy-coat-depleted EC on day 0.*
Figure 4/A: *differential acquisition of PI-stained nuclei.*
Figure 4/B: *discrimination of nuclei by nucleus complexity (SSC).*
[Nuclear analysis using the different patterns of side-scatter (SSC) of polynuclear and mononuclear nuclei allows differentiation between the two populations. As shown in Figure 4/A, the prospectively decaying population of residual cells in EC is composed mainly of polynuclear WBCs, since these cells are clearly discriminated from mononuclear cells based on SSC pattern (Figure 4/B).]

Statistical considerations on quantification methods and random sample control

The stringent product specification, especially regarding rWBCs (< 1 x 10^6 to 5 x 10^6 per unit)[3, 4, 26] define the maximal acceptable rWBC concentration as 4-10 rWBCs/µL in leucodepleted blood products depending on the final volume of the product. Given the sample preparation procedure and the limited sample volume processed by the quantification device, the number of acquired events for calculation of the final rWBC concentration in the product is usually < 50 events[10]. Given a homogenous distribution of rWBCs in the leucoreduced product and a technically ideal sample drawing, the probability of acquiring the sample's rWBC completely is governed by the Poisson distribution[28]. The statistical error of enumeration is therefore given by the formula:

$$\% \text{ Error} = 100 \times \frac{\{1{,}96 \times \sqrt{C}\}}{C}$$

where C = number of events counted.

Using this formula, one can estimate the inherent enumeration error, which will largely define the accuracy of the final result[14]. Figure 5 shows the theoretical progression of enumeration error depending on the acquired events. Note that the error is independent of the enumeration method used and can be improved by increasing the captured events. Dzik[11] showed, in a multicentre study comparing FACS and Nageotte haemocytometry, that because of the low number of events counted in leucodepleted blood products, the microscopic quantification technique behaves at best like a non-parametric measurement method (failed/pass discrimination) at the requested rWBC specification level. In contrast, FACS quantification is accurate enough to allow parametric evaluation of rWBC in leucodepleted products. Therefore, to obtain the same confidence in data, one would have to examine 10-20 times more units by Nageotte haemocytometry than by FACS.

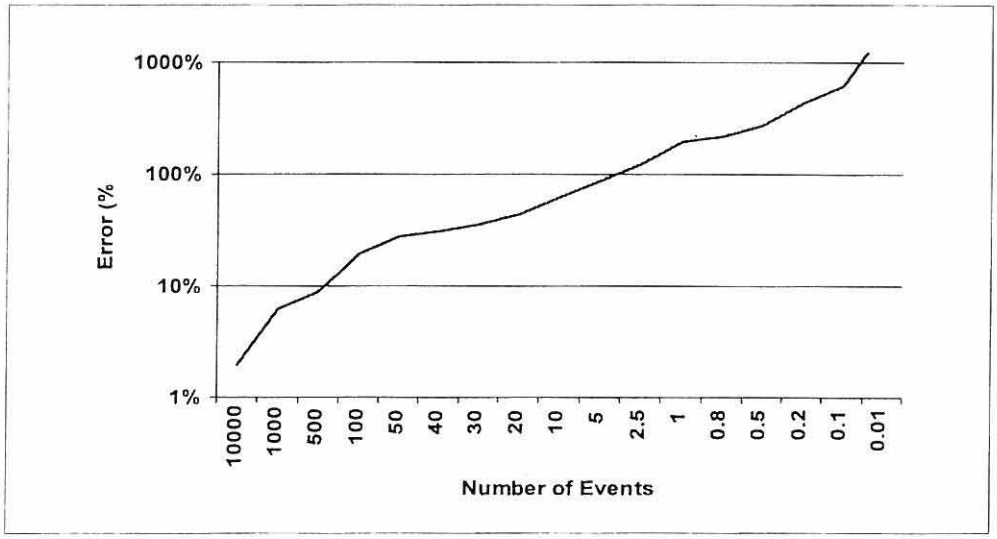

Figure 5 - *Theoretical progression of error by Poisson probability.*
[The theoretical progression of error in % was calculated as a function of observed/acquired events using the formula indicated (see text). Note that the progression of error is determined only by the number of observed events (for discussion see text).]

Various methods to improve the sensitivity and accuracy of rWBC enumeration have been suggested[18, 29, 31, 32]. Earlier attempts focused on the enrichment of rWBC by sample centrifugation or by gradient centrifugation of the entire blood product. However, such approaches introduce new, poorly controlled sources of errors (cell loss and disproportional cell enrichment) or are destructive to the product. Recently, Backteman *et al.* presented data showing that simply by increasing the neat sample volume to the limit of proper propidium iodide staining of DNA and using standardised TruCount® tubes, which do not require dispensation of microbeads, the sensitivity and accuracy of flow-cytometric quantification of rWBC can be substantially improved[5].

Another important issue that must be considered when defining quality control of leucodepletion processes is the type of assessment of the process failure that might occur. Assuming log-normal distribution of rWBC in leucodepleted blood products [20], there are two different types of possible process failure. First, a systematic failure may occur, af ecting all units produced and resulting in deterioratiorf of the mean rWBC (unimodal distribution shift). Second, there may be an admixture of failed products with a significantly aberrant rWBC content, which produces a different log-normal distribution as compared to that of the compliant population (bimodal distribution shift) [1]. The mean is a powerful descriptor for detecting drifts in unimodal distribution, especially when the variance of the mean is low. Even small numbers of units tested (4-10) are capable of detecting process deterioration with a high level of confidence. If the variance is high, considerably larger numbers of units (> 20) must be tested to provide the same confidence [1]. In addition, the mean of randomly chosen samples from a log-normal distribution reliably detects a minimal drift of the population, even before the drift has resulted in a product failure.

The bimodal distribution of process failure is much more difficult to handle. A bimodal distribution might occur as a consequence of failure of a single processing device (e.g. a centrifuge, an apheresis machine or a description violation by a single employee). Bimodal distributions would best be evaluated by examination of large samples, or even the whole batch of units produced, for the presence of individual outliers (non-parametric examination). In the computer model by Adams, a sample of 100 tests would be necessary to detect 5% outliers with a confidence of 99% in a population of 1.000, and a sample of 500 would be necessary to detect 1% outliers with the same confidence [1]. Similarly, if the failure occurs sporadically, the likelihood of detecting a violation by random sampling, such as 1% of production, is small. This is due simply to sampling error. Therefore, if a bimodal distribution of process failure is suspected, it may be worth considering temporarily changing the established practice of regular sampling [1].

Practical guidelines for validation, implementation and ongoing quality control of leucodepletion have been produced [2, 9]. According to the Biomedical Excellence for Safer Transfusion Working Party (BEST), the successful local implementation of a new process should be demonstrated by checking at least 20 consecutive units, if a parametric evaluation is chosen. In the case of a non-parametric measurement (failed/pass discrimination), a minimum of 60 units must be checked [9]. Once the process has passed validation examinations, ongoing process controls should be applied using Levey-Jennings type controls [22] to demonstrate process stability.

Conclusions

The implementation of stringent product specifications following improved processing techniques of donated whole blood has prompted the revision of quality assurance of leucodepleted blood products. While traditional enumeration techniques such as microscopic counting methods are inexpensive and widely accessible, newer cell quantification technology based on flow-cytometry has proven to be superior in terms of accuracy, reliability, robustness, data availability and documentation. In addition, high sample throughput and the potential to adapt protocols to specific needs are very

welcome advantages of FACS-based methods. Although FACS-based techniques are demanding in terms of resources, these techniques economise quality assurance of leucodepletion processes.

Accurate quality control of leucodepletion processes needs to be based on statistical rules and mathematical modelling, which constitute the basis of statistical process control [34]. When defining frequency and number of units to be investigated, one must be aware of applicable statistical theories as well as of all the key factors which have the potential to cause deterioration of the process outcome.

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