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Comparative analysis of three permeabilization methods for cytofluorometric evaluation of cytoplasmic myeloperoxidase

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Abstract

A comparative study was conducted to evaluate three different permeabilization methods: FACS Permeabilizing Solution (FPerm), CytoFix/CytoPerm Kit (CFP) and Paraformaldehyde-Tween 20 (PFT) reagents, in cytoplasmic labeling of myeloperoxidase (MPO). Peripheral blood cells from 23 healthy subjects were fixed and permeabilized according to the proposed procedures, prior to direct immunofluorescence staining with CD14, CD45, IgG_1, IgG_2 and MPO monoclonal antibodies (McAb). Subsequent flow cytometric analysis was performed on FACSCalibur flow cytometer (Becton Dickinson, BD). As far as the antigenic expression of MPO in normal samples is concerned, FPerm and CFP demonstrated better cytoplasmic staining by inducing minor effects on light-scattering properties of the cell populations, whereas PFT-treated samples showed a diminished ability to distinguish the cell types. However, the simple and rapid FPerm method required an earlier processing of samples since the stored whole blood samples (for more than 8 hours) tended to show a significant decrease of fluorescence intensity. We also have demonstrated that P/N ratio possesses added value in evaluation of cell reactivity in immunophenotyping, based upon the apparent nonspecific cytoplasmic staining of MPO in the lymphocyte population.

Key words: FACS Permeabilizing solution, CytoFix/CytoPerm, paraformaldehyde-Tween 20, fixation, permeabilization, cytoplasmic myeloperoxidase.

INTRODUCTION

Flow cytometry is emerging as an important adjunctive technology in the delivery of contemporary medicine. In recent years, immunophenotyping of leukaemic cells with vastly available commercial McAb has become a generally accepted part of the work-up of a patient with acute leukaemia, which supplements morphologic, cytochemical and cytogenetic methods that lead to the best diagnosis of leukaemias. In the characterization of acute leukaemias, flow cytometric analysis in many ways remains the preferred approach to define the immunophenotype of the malignant cells due to the efficiency with which a large repertoire of cellular markers can be examined and the desirability of examining coexpression of multiple markers.[1]

As several of the antigens detected in blast cells are expressed first in the cytoplasm at an earlier stage of differentiation than on the membrane, a comprehensive immunophenotype requires the detection not only of surface antigens (*s*Ag) but also of intracellular antigens (*i*Ag).[2,3,4] Hence, development of fixation and permeabilization procedures for the detection of *i*Ag without altering *s*Ag expression possesses a great value in immunophenotyping of acute leukaemias. Over the last decade, few techniques have been described to give satisfactory *i*Ag labeling, by employing different fixatives and detergents in the fixation and permeabilization procedures. For instance, the use of buffered formaldehyde acetone,[5] digitonin,[6] lysolecithin,[7] saponin,[8] Tween 20,[9] paraformaldehyde and ethanol.[10] Currently, it has been shown that various commercial red cell lysing methods can be used for the permeabilization of either separated or unseparated leukocytes. However, in their recent studies, Tiirikainen[11] and Lanza *et.al.*[12] had demonstrated that, among the available commercial red cell lysing solutions, only the FACS lysing solution (BD) was applicable for complete permeabilization of leukocytes and for the study of *i*Ag alone or simultaneously with the *s*Ag.

Based on these data and on the current availability of various fixation and

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permeabilization kits, it appears to us interesting to compare our in-house method (PFT) with two commercially available permeabilizing solutions (FPerm and CFP) for the analysis of cytoplasmic MPO, which has proven to be of major importance in immunophenotyping of acute leukemic blasts. Besides, the storage effect on the fluorescence intensity of MPO expression was also studied.

MATERIALS AND METHODS

Blood samples

Three sets (for three permeabilization methods) of EDTA-anticoagulated peripheral blood (PB) were obtained from each of the 23 healthy adult individuals. Each set consist of two tubes and each tube from each respective set was processed within 2 hours while the remaining tubes were held at room temperature for more than 8 hours. For FPerm, lysed whole blood (LWB) samples were prepared from incubation of 100 p1 PB with 2 ml of 1X FACS lysing solution (BD) at room temperature for 10 minutes. The cells were then pelleted by centrifuging at 500*g* for 5 minutes. Meanwhile, mononuclear cells (MNC) were isolated by density gradient centrifugation with Lymphoprep (Nycomed, 1.077 g/ml). Interphase cells were aspirated and washed twice with phosphate buffered saline (PBS) pH 7.4.

FACS permeabilizing method

LWB cell pellet was subjected to fixation and permeabilization by incubating in 500 µl 1X FACS Permeabilizing solution (BD) for 10 minutes at room temperature in the dark. After two washes with PBS supplemented with 0.5% bovine serum albumin (Sigma) and 0.1% sodium azide (Sigma), the cells were stained with McAb. Then, the washing steps were repeated before resuspending cells in PBS for flow cytometric analysis.

CytoFix/CytoPerm method

This method was carried out according to the proposed procedure (Pharmingen, BD). In brief, 100 µl MNC were first fixed and permeabilized in 250 µl CytoFix/CytoPerm solution for 20 minutes at 4 °C in the dark. After washing with 1X Perm/Wash solution, cells were resuspended in 50 µl Perm/Wash solution and proceeded to McAb staining. Then, the stained cells were washed twice with Perm/Wash solution before resuspended in PBS for flow cytometric analysis.

Paraformaldehyde-Tween 20 method

400 µl MNC were fixed in 875 µl cold PBS and 125 p1 2% paraformaldehyde (Sigma) for 1 hour at 4 °C in the dark before permeabilized 15 minutes with 1 ml 0.2% Tween 20 (Sigma) at 37 "C. Following addition of 1 ml of 2% foetal calf serum (FCS, Gibco BRL) in 0.1% sodium azide in PBS, cells were pelleted and resuspended in FCS. The susupension was then divided into 100 µl each for immunostaining. After 2 washes with 0.2% Tween 20, the cells were resuspended in PBS for flow cytometric analysis.

Immunofluorescence staining

Suspension stainings of *i*Ag were performed by subsequently incubating the fixed and permeabilized cell pellet with 10 µl of the appropriate fluorochrome-conjugated McAb for 30 minutes at 4 °C in the dark: CD14/CD45 (Leucogate, BD) and MPO (MPO-7, Dako). Isotypic specific negative control stainings were included: CD14 (isotype IgG_2, BD), CD45 (isotype IgG_1, BD) and MPO (isotype IgG_1, BD).

Flow cyrometric analysis

Stained cells were applied to the modular benchtop FACSCalibur flow cytometry (BD) equipped with a 15 mW air-cooled 488 nm argon-ion laser. Data acquisition and analysis were performed using SimulSET (version 3.1) and CELLQuest (version 3.0) softwares. A total event of 15 000 cells were acquired for each sample. Two of the main blood cell populations, lymphocyte (L) and monocyte (M) were identified by light-scattering parameters, i.e. forward *vs.* side-angle scatter. Data were expressed as geometric mean fluorescence intensity and as ratio between the fluorescence emission of sample cells and that of the isotypic control (P/N ratio; positive/negative).

Statistical analysis

For comparison among the three permeabilization methods, the geometric mean fluorescence intensity of each intracellular marker were subjected to ANOVA analysis. Whereas, paired T-test was employed to evaluate the storage effect on the intensity of cytoplasmic antigen expression.

RESULTS

Table 1 shows the mean values of geometric

mean fluorescence intensity and the P/N ratios (calculated as described by Lanza *et.al.*[12,13]) in the two blood populations (L and M). Referring to the results presented in Table 1 and Figure 1, although all the three methods were capable of permeabilizing the leukocyte population, they were characterized by a lower specificity in detecting cytoplasmic MPO. A clear-cut positivity was also observed in the vast majority of L, which is a cell type devoid of those granular constituents of myeloid cells. These findings coincide with those presented by Lanza *et.al.*[14] This nonspecific staining was not due to the concentration of McAb used; it was also

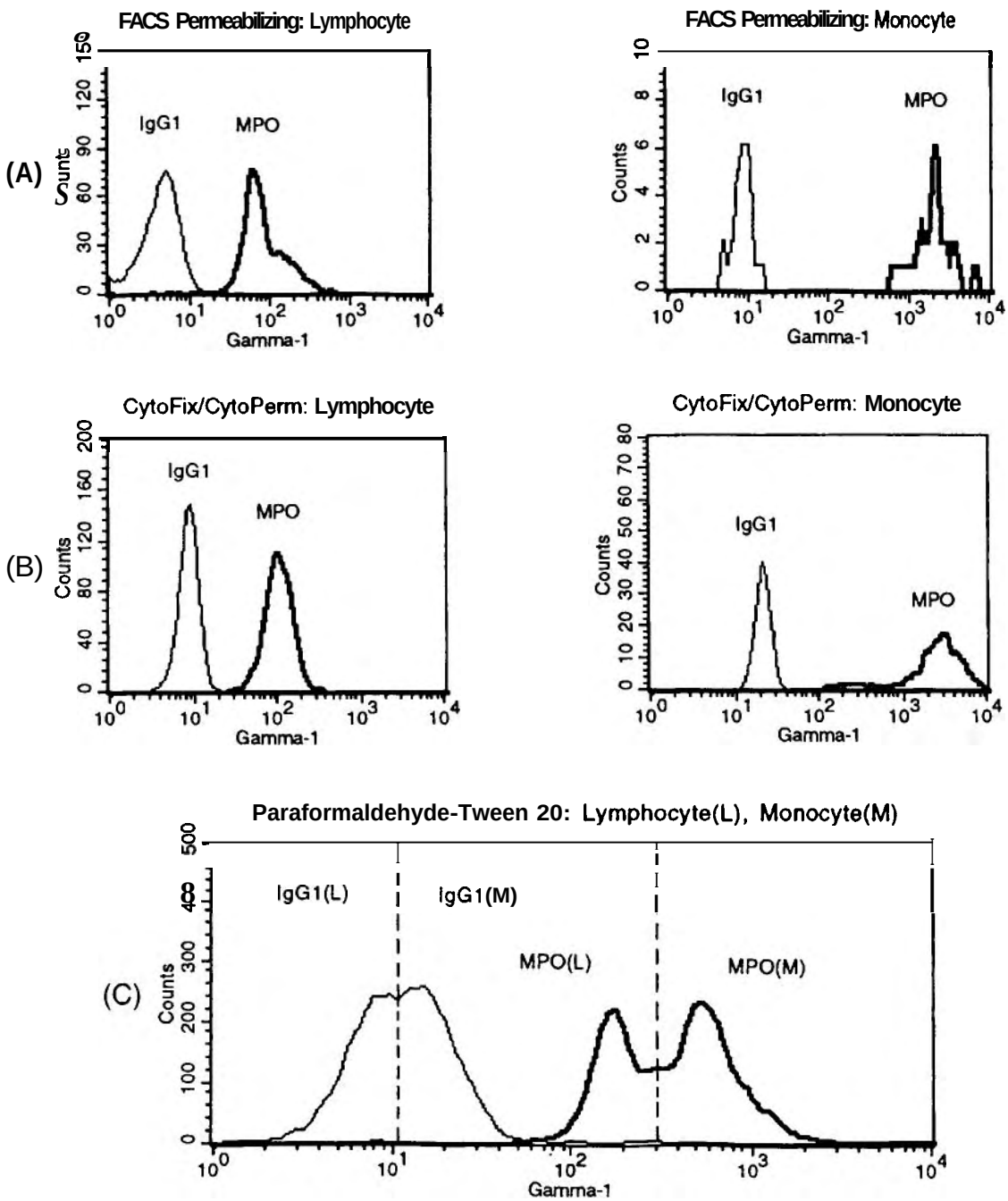

FIG. I: Flow cytornetric analysis of cytoplasmic rnyeloperoxidase (MPO) utilizing: (A) FACS permeabilizing solution, (B) CytoFix/CytoPerm **Kit, (C) Paraformaldehyde-Tween 20 reagents.**

TABLE 1: Flow cytometric analysis of cytoplasmic CD14, CD45 and MPO in blood cells from 23 healthy individuals using 3 different fixation and permeabilization methods[a].

Parameter	CD14			CD45			MPO		
	FPerm	CFP	PFT	FPerm	CFP	PFT	FPerm	CFP	PFT
Fluorescence Intensity									
Lymphocyte	2.9 ± 1.0 (1.7 – 4.7)	2.7 ± 0.3 (2.2 – 3.4)	3.5 ± 1.9 (1.8 – 8.5)	383.2 ± 147.2 (160.9 – 544.0)	434.1 ± 115.5 (191.4 – 634.6)	173.8 ± 73.7 (61.0 – 350.5)	43.7 ± 24.7 (14.9 – 91.5)	52.6 ± 36.5 (9.9 – 123.8)	92.2 ± 60.3 (24.5 – 259.9)
Monocyte	998.6 ± 881.5 (116.8 – 3071.0)	410.3 ± 197.2 (89.7 – 930.2)	16 ± 141.8 (.2– 61.3)	291.9 ± 56.9 (174.9 – 447.6)	66.5 ± 50.6 (24.2 – 189.1)	60.3 ± 29.3 (12.2 – 132.4)	958.2 ± 512.6 (339.1 – 2015.0)	2191.2 ± 998.8 (287.9 – 3837.7)	649.0 ± 195.7 (103.1 – 1020.7)
P/N Ratio									
Lymphocyte	0.5 ± 0.1 (0.3 – 0.8)	0.5 ± 0.1 (0.3 – 0.8)	1 ± 0 .4 (0 – 2 .6)	112.6 ± 49.6 (47.4 – 190.2)	56.2 ± 11.1 (32.9 – 77.2)	55.8 ± 37.5 (5.7 – 103.5)	12.5 ± 6.8 (4.1 – 23.9)	6.6 ± 4.2 (1.6 – 15.5)	22.0 ± 18.0 (6.6 – 97.0)
Monocyte	57.0 ± 29.5 (15.5 – 108.7)	17.7 ± 7.7 (6.9 – 38.4)	3.5 ± 11.9 (1.0 – 3.4)	44.3 ± 10.8 (20.3 – 64.6)	3.4 ± 2.6 (1.0 – 10.3)	3.0 ± 1.8 (0.1 – 7.3)	138.8 ± 63.5 (56.7 – 286.6)	111.4 ± 52.5 (18.3 – 193.4)	31.7 ± 15.4 (4.6 – 59.0)

[a] Data are expressed as: Fluorescence intensity – geometric mean fluorescence intensity ± 1 SD (range); P/N ratio – mean ratio ± 1 SD (range).

TABLE 2: Storage effect on cytoplasmic expression of CD14, CD45 and MPO using 3 different fixation and permeabilization methods[b].

Antigen	FPerm		CFP		PFT	
	I	II	I	II	I	II
Lymphocyte						
CD14	3.8 ± 0.6*	2.9 ± 0.5*	2.6 ± 0.3	2.7 ± 0.2	5.2 ± 1.6	6.7 ± 2.1
CD45	225.4 ± 45.4*	189.9 ± 33.3*	518.0 ± 55.9	500.6 ± 54.4	107.9 ± 42.0	182.4 ± 144.7
MPO	69.1 ± 11.9*	55.4 ± 12.5*	73.3 ± 33.4*	112.7 ± 42.6*	132.0 ± 28.7	160.5 ± 76.0
Monocyte						
CD14	1832.4 ± 702.8*	935.7 ± 551.8*	445.0 ± 118.0	477.1 ± 158.7	56.1 ± 28.3	65.9 ± 65.4
CD45	319.7 ± 55.1*	247.5 ± 62.0*	92.6 ± 67.9	88.9 ± 67.3	39.3 ± 20.3	51.2 ± 33.0
MPO	1399.0 ± 432.7	1236.4 ± 609.7	2987.9 ± 495.1	2895.0 ± 381.4	556.7 ± 107.1	763.0 ± 375.0

[b] Data are expressed as geometric mean fluorescence intensity ± 1 SD.
* The mean difference is significant at the 0.05 level.
Samples were processed within 2 hours (I) or after 8 hours (II) of collection.

observed in cell samples incubated with suboptimal McAb concentrations.[14] However, fluorescence intensity of positively stained L was still markedly lower as of M, except for the PFT method (Table 1). As shown in Table I, different methods gave different cut-off value for cytoplasmic MPO staining. Therefore, it was determined that only values for the P/N ratios higher than 23.9 (FPerm); 15.5 (CFP); 97.0 (PFT) should be regarded as an indicator of cell positivity.

Although a number of methods exist for intracellular labeling, most of them result in significant changes to the light scatter profiles of MNC such that whole blood (WB) samples must be lymphopreped prior to treatment.[9,15] This added step results in extended processing time and more critically, the potential for cell loss. Of the three methods evaluated, only FPerm could be applied on WB samples. On the other hand, our comparative study demonstrated that WB samples were inadvisable to be processed after 8 hours of drawing since the fluorescence intensity tended to dim significantly as determined by paired T-test analysis (Table 2). Interestingly, a significant increase of MPO expression was also revealed in CFP-treated samples (Table 2).

As can be seen from Figure 2, FPerm and CFP methods had only minor effects on light scatter properties of the cell populations, as L and M were clearly discernible as two defined populations. CFP-treated samples gave a clearer background as very little debris or cell aggregates were presented. On the contrary, the recognition of M population was hardly achievable using PFT method, a partial overlapping between L and M was noticed.

DISCUSSION

Nowadays, simultaneous cytofluorometric evaluation of *s*Ag and *i*Ag of leukaemic cells with McAb has been proven useful and essential in the diagnosis and classification of acute leukaemias. Nevertheless, it should be borne in mind that the pattern of reactivity for each McAb directed against an *i*Ag should be carefully evaluated in many cell types before making any diagnostic and prognostic assumptions regarding acute leukaemias.[16]

Of note, the methodology employed for the study of *i*Ag is critical for a precise interpretation of cytofluorometric data. Applications requiring accurate quantitative measurements of *i*Ag may require different types of controls to assure the quality of the data. Fixed cells have different levels of background fluorescence, depending on the type of cell and the techniques used that will be increased by nonspecifically-bound fluorochrome in the cell.[17] During fixation and permeabilization procedures, preferential cell losses often occurred apparently due to repeated cell washes and centrifugation.[18] Utilization of

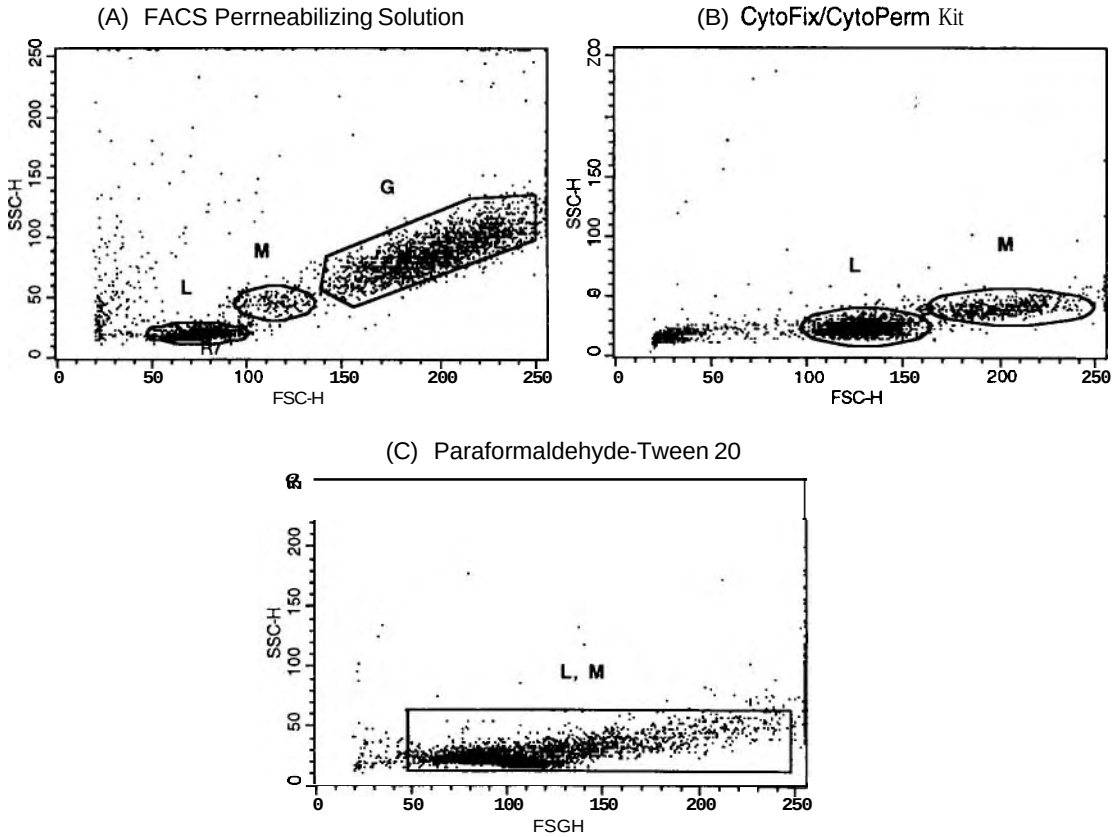

FIG. 2: Effects of three different fixation and permeabilization methods on light scatter properties of the cell populations. (L= lymphocyte; M= monocyte; G= granulocyte).

various fixatives and detergents has led to morphologic damage, cell aggregation or loss of intracellular antigenicity,[19] which hampered satisfactory intracellular labeling. For instance, Tween *20* in concentrations higher than *0.5%* caused disruption of the scatter properties, so did paraformaldehyde in concentrations of less than 2%.[20]

Our findings are in line with those presented by Lanza *et.al.*[14], confirming the necessity of a careful evaluation of the effectiveness and specificity of a given permeabilization method before it is proposed to be implemented as a routine basis. Comparing to our in-house (PFT) and CFP methods, the capability of direct analysis of unseparated WB makes FPerm method simple with minimal handling of samples. Hence, this rapid technique should be a valuable addition to routine study of intracellular antigens on the condition that WB samples must be analyzed within 8 hours of collection.

On the other hand, taking into account the nonspecific staining of L population, parameter P/N ratio as presented by Lanza *et.al.*[12,13] may be of great value for the evaluation of cell reactivity in analysis of peripheral blood leukocytes.

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