Eur Respir J 2004; 24: 309–312
DOI: 10.1183/09031936.04.00125603
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European Respiratory Journal
ISSN 0903-1936

Effect of freezing of sputum samples on flow cytometric analysis of lymphocyte subsets

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ABSTRACT: Sputum samples should be processed shortly after induction to prevent cell degradation. For intermediate storage, freezing of homogenised samples or immediate fixation have been shown to be suitable for cytospins. The aim of this study was to investigate whether freezing or immediate fixation of sputum affect the analysis of lymphocyte subsets by flow cytometry.

Selected plugs from 24 sputum samples were homogenised. One aliquot was processed immediately and analysed by flow cytometry. A second aliquot was homogenised, frozen at -20°C after addition of dimethylsulfoxide and stored for a median time of 6 days. In six samples a third aliquot was fixed in formalin after induction and stored for up to 72 h before further processing.

Compared to immediate processing, percentages of total lymphocytes and T-suppressor cells were elevated after being frozen, with a minor decrease in the T4/T8 ratio. Proportions of total lymphocytes, T-helper and T-suppressor cells correlated between native and frozen samples, intra-class correlation coefficients being 0.74, 0.85 and 0.70, respectively. The formalin-fixed aliquots could not be analysed with the antibodies used.

In conclusion, freezing seems to be a suitable technique to store sputum samples for flow cytometry of CD3, CD4 and CD8 lymphocyte subsets. Its effects were minor compared to the variation between subjects.

Eur Respir J 2004; 24: 309–312.

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Keywords: Fixation, flow cytometry, freezing, lymphocyte subsets, sputum induction

Received: November 10 2003
Accepted after revision: April 14 2004

This study was supported by Landesversicherungsanstalt (LVA) - Freie und Hansestadt Hamburg and Labor Dr. Kramer und Kollegen, Geesthacht, Germany.

Sputum cells can be analysed by flow cytometry to obtain data on lymphocyte subsets [1, 2] and intracellular cytokine production [2]. For differential cell counts on cytospins it is recommended that sputum be processed soon after expectoration to prevent degradation of cells [3]. Freezing or fixation of sputum has been suggested to elongate time until processing, allowing, for example, transport to a specialised laboratory [4, 5]. This aim of this study was to assess the effect of intermediate storage on flow cytometric analysis of lymphocyte subsets using the freezing and the fixation methods [4, 5].

Materials and methods

Subjects

A total of 24 sputum samples from 18 subjects were analysed. Six subjects were healthy (two males/four females; mean±SD age 28±6 yrs; forced expiratory volume in one second (FEV1) 106±15% predicted), nine had asthma of different severity (five males/four females; age 40±11 yrs; FEV1 73±14% pred) and three had allergic rhinitis without asthma (3 males; age 32±7 yrs; FEV1 110±17% pred). All subjects gave their informed consent and measurements were approved by the local Ethics Committee. Sputum inductions were performed by inhalation of 3-5% or 0.9% saline. Selected plugs were separated into two aliquots immediately after homogenisation with Sputolysin® (20 min at room temperature, 6.5 mM dithiothreitol; Calbiochem, Darmstadt, Germany) [6, 7]. One aliquot was mixed 1:1 with PBS containing 1% BSA and 30% dimethylsulfoxide (DMSO; Sigma, Munich, Germany), and stored frozen at -20°C (median storage time 6 days (range 1–16)). The second aliquot (native sputum) was processed as usual [6].

In six samples a third aliquot was taken before homogenisation and processed with the method proposed by KELLY *et al.* [5], but with formalin as fixative. In short, 10 mL of neutral buffered formalin was added and the mixture briefly vortexed. The sample was stored at room temperature for a median time (range) of 64 h (54–78). After this it was centrifuged at 300×*g* for 10 min and washed three times with 10 mL PBS. A freshly thawed solution for enzymatic dispersal [5] was added (3 μL to 1 mg of sputum), briefly vortexed and incubated at 37°C for 6 h before flow cytometry analysis.

Flow cytometry

A FACSCalibur® (BD Biosciences, Heidelberg, Germany) was used for four-colour flow cytometry. Cells were adjusted to 0.5×10^6 cells·mL^{-1} in PBS containing 1% BSA. Three aliquots were taken and measured using monoclonal antibodies for direct immunofluorescence (MultiTest®; BD

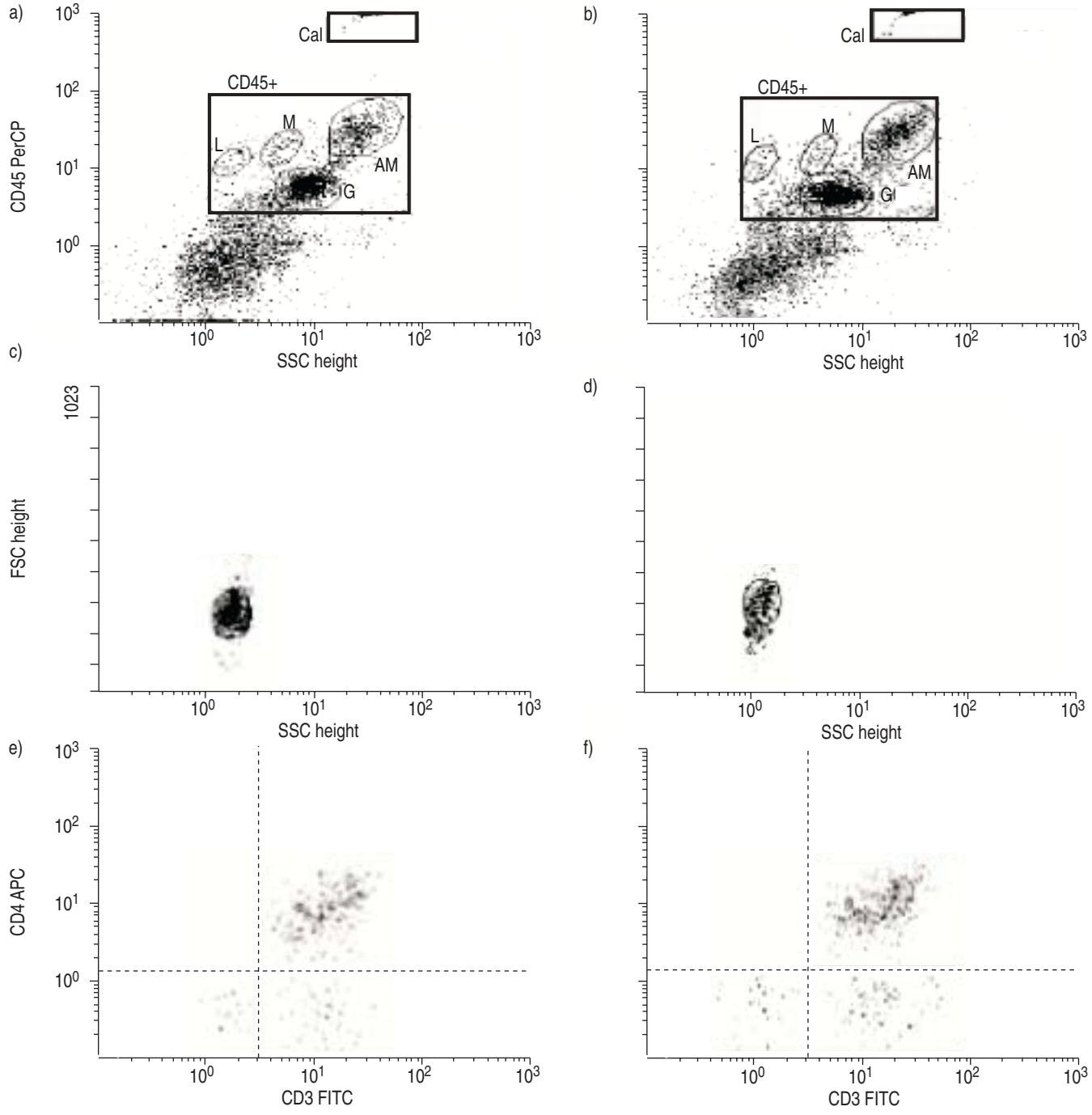

Fig. 1. – Example of a flow cytometric analysis from a representative sample (from one subject), with plots on the left (a, c, e) showing data from a native and plots on the right (b, d, f) data from frozen aliquots of one sputum sample. Plots a) and b) show CD45 binding *versus* sideward scatter (SSC), c) and d) forward scatter (FSC) *versus* SSC, and e) and f) CD4 *versus* CD3. Plots a) and b) illustrate the first step of gating, with AM indicating alveolar macrophages, M monocytes, G granulocytes and L lymphocytes. CD45-bright lymphocytes were selected to identify morphologically intact lymphocytes (c, d). Cells from plots c) and d) were then used to define CD4+ cells (e, f) *versus* CD3. PerCP: peridinin-chlorophyll protein; Cal: calibration beats; APC: allophycocyanin; FITC: fluorescein isothiocyanate.

Biosciences). Antibody panel I comprised CD45 (leukocytes) and propidium iodide (PI; viability), panel II CD3/CD8/CD45/CD4 (mature T, suppressor/cytotoxic, helper/inducer T-cells) and panel III CD3/CD16+CD56/CD45/CD19 (natural killer (NK), B-cells). Native samples were incubated with fluorescence-labelled antibodies according to the manufacturer's instructions. Subsequently, 0.4-mL FACSFlow® (BD Biosciences) was added. In a subset of 16 samples, FlowCount® Fluorospheres (Beckman Coulter, Krefeld, Germany) were added after incubation with the antibodies to derive absolute cell number. Frozen cells were treated in the same manner, after they had been quickly thawed, dispersed in 4 mL CellWASH® (BD Biosciences) and centrifuged at 500×*g* for 5 min. After two more washing steps, cells were resuspended in 350 μL CellWASH®.

Lymphocytes were defined *via* CD45 and sideward scatter (SSC); their population showed the low SSC and an elongated width of forward scatter (FSC) characteristic of sputum [8]. To obtain mainly morphologically intact cells, and to avoid degraded cells and debris, this population was gated in the

more upper part of the FCS distribution and checked for an optimum of cell viability *via* PI dye exclusion (panel 1). Lymphocyte subpopulations were identified on the respective bivariate plots (fig. 1).

Statistical analysis

Comparisons were performed by the Wilcoxon test. Reproducibility was quantified by intraclass correlation coefficients (ICC). To check the consistency of results, 100% minus the sum of the percentages of T-, B- and NK cells (checksum 1), and the percentage of CD3+ cells minus the sum of CD3+/CD4+ and CD3+/CD8+ cells (checksum 2) was computed. Statistical significance was assumed at $p<0.05$.

Results

Numbers of morphologically intact lymphocytes, as judged by light scatter characteristics, were slightly reduced in frozen compared to native aliquots (geometric mean (SD) 0.048 (3.1) *versus* 0.036 (2.5) $\times 10^6$ cells·mL^{-1}). Irrespective of this, a well distinguishable lymphocyte population could be recognised in all samples, enabling a straightforward further gating (fig. 1).

The agreement between native and frozen samples was acceptable, particularly for CD4 and CD8 (fig. 2). Intraclass correlation coefficients for CD3, CD4 and CD8 were $\geqslant 0.70$ (table 1). There was no obvious relationship between the time of storage and the quality of cells. None of the formalin-fixed samples could be analysed by flow cytometry, as there were no interpretable signals in the expected range of fluorescence.

Table 1. – Percentages of sputum cells, intraclass correlation coefficients (ICC) and Spearman rank correlation coefficients (R_s)

	Native	Frozen	ICC	R_s
CD3+	92.3 (89.2–95.8)	96.2 (91.1–97.2)**	0.74	0.68
CD4+	77.1 (64.2–80.3)	75.6 (67.6–79.6)	0.85	0.70
CD8+	15.0 (11.6–20.4)	19.3 (14.6–22.6)**	0.70	0.83
T4/T8	5.0 (3.2–6.8)	3.9 (2.9–5.5)*	0.77	0.80
CD19+	3.0 (1.4–6.4)	2.0 (0.8–4.9)	0.79	0.75
CD16/56+	1.3 (0.6–1.7)	1.5 (0.3–2.0)	0.13	0.22
Viability	76.5 (59.1–84.9)	63.9 (42.1–73.8)	0.33	0.29
Checksum 1	2.1 (0.4–3.1)	1.0 (-0.1–1.8)		
Checksum 2	1.1 (-1.4–2.3)	0.4 (-1.7–1.5)		

Data are presented as median (quartiles); Percentages of CD3-positive cells and lymphocyte subsets refer to the lymphocyte gate (forward scatter *versus* sideward scatter, morphologically intact cells). *: $p<0.05$; **: $p<0.01$ according to the Wilcoxon matched-pairs signed-ranks test.

Discussion

The data from this study suggest that freezing for intermediate storage of induced sputum has no major effects on the analysis of CD3, CD4 and CD8 lymphocyte subsets by flow cytometry. Irrespective of minor differences in median values, the correlation between native and frozen samples was

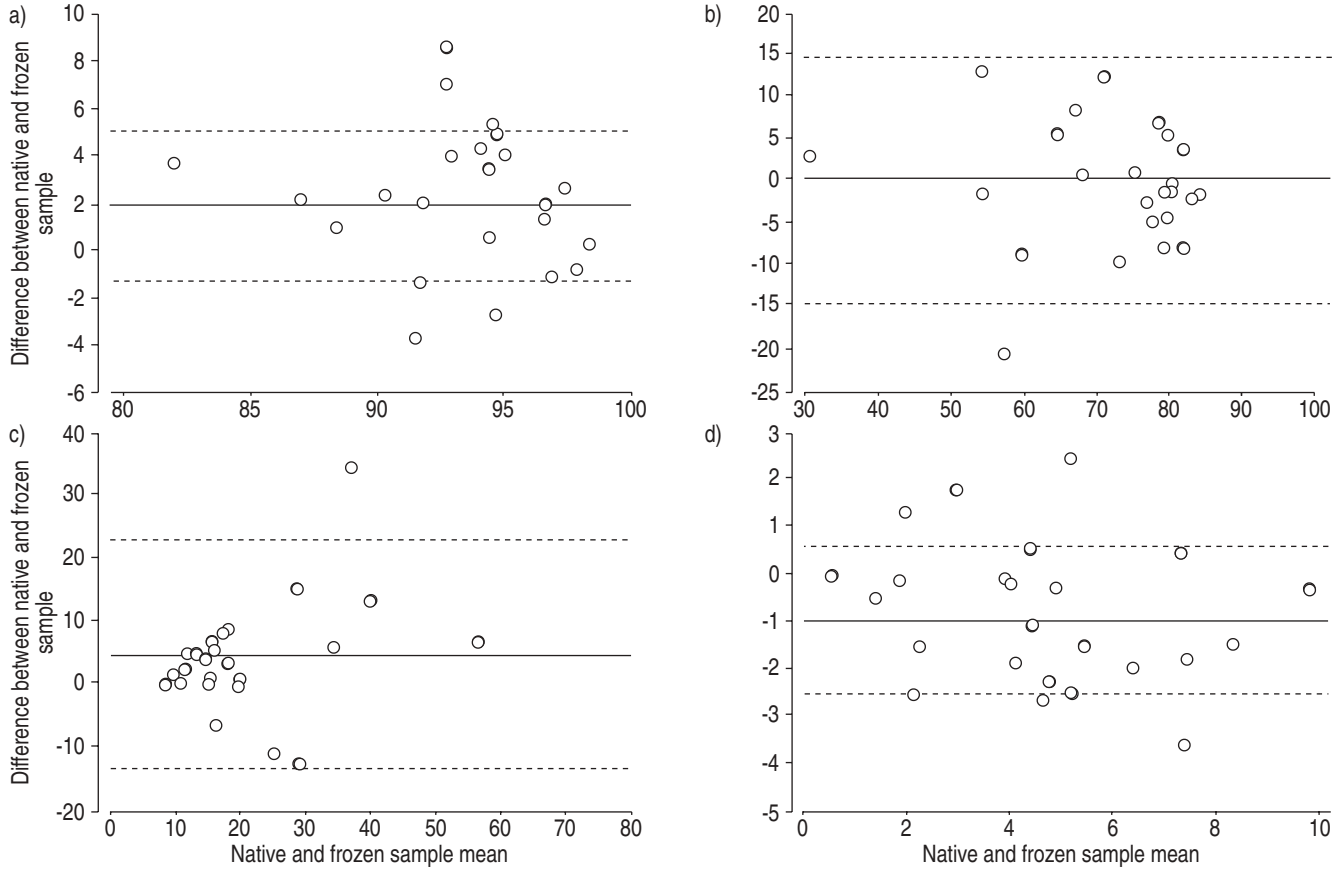

Fig. 2. – Bland-Altman plots to show the relationship between mean values of measurements and differences between measurements obtained in frozen and native samples. Note that the larger scatter observed for a) CD3 and d) T4/T8 compared to b) CD4 and c) CD8 is related to the smaller range of variation of these variables. Corresponding intraclass correlation coefficients are given in table 1.

high, particularly with regard to these major subsets of interest.

Since frozen cells are fragile, all samples were handled as gently as possible during thawing and mixing. There was only a minor loss in the total number of morphologically intact lymphocytes that were included in the immunological gate. Apparently, the lower number did not affect the correlation between native and frozen aliquots, indicating that potential selective losses were not relevant. Differences in the pattern of morphologically intact cells (fig. 1a–d) were probably related to the lack of correlation of viability between frozen and native aliquots; despite this, median values were similar (table 1). The slightly elevated numbers of CD3+ and CD8+ cells, plus the concomitant reduction in T4/T8, were probably due to shifts in immunological gating as a result of shifts in morphological gating. However, additional differential effects of freezing on CD4+ and CD8+ cells cannot be excluded. Importantly, the identification of CD4+ cells seemed to be unaffected by freezing and median values of lymphocyte subsets (table 1) corresponded well to published data [1]. In addition, the checksums were close to expected values, indicating a reasonable gating in both native and frozen aliquots.

In addition to freezing, the effect of a recently described sputum fixative [5] was assessed on the determination of lymphocyte subsets. The use of a fixative could further simplify the logistics of transportation as it does not require a cooling chain. Using this approach, however, the formalin-fixed samples could not be analysed by flow cytometry, probably due to a lack of binding of the antibodies. The relevant antigens may have been formalin-sensitive or masked by formalin; epitopes of CD4, CD16, CD19 and CD56 are destroyed by formalin fixation; CD3, CD8 and CD45 require antigen retrieval. Whether flow cytometry of formalin-fixed samples can be achieved by different antibodies or adjustments in the processing steps remains to be evaluated. In this respect the procedure in which antibodies are added first followed by fixation of the samples deserves further exploration.

In summary, the present data suggest that freezing of sputum with 15% dimethylsulfoxide at -20°C is suitable for storage over ~1 week and does not markedly affect the flow cytometric analysis of CD3, CD4 and CD8 lymphocyte subsets. Thus, freezing might facilitate the usage of sputum analysis in multicentre clinical trials, as well as clinical practice.

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