Analysis of the Temperature Affects on Leukocyte Surface Antigen Expression

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Flow cytometric analysis of leukocyte surface antigens has been used to characterize infectious and septic processes in patients. We wanted to investigate how the sampling and processing temperature, the anticoagulant used, and the storage of the sample influence leukocyte immunophenotyping. Four blood samples, two using acid citrate dextrose and two using heparin as an anticoagulant, were taken from five intensive-care unit patients with severe sepsis and five healthy volunteers. The samples were collected, stored, and processed either at +4°C or at room temperature (RT). The samples were processed for flow cytometric analysis within 1 hr of collection or after 6 or 24 hr storage.

The surface antigens of interest were neutrophilic CD11b and CD64, monocytic CD11b, CD14, CD40, CD64, CD80 and HLA-DR, and lymphocytic CD69 (separately in CD4+ and CD8+ T cells, B cells, and natural killer cells). The fluorescence intensities were higher at RT than at +4°C. During storage the intensities increased at RT, but at +4°C there were only minor changes. The effects were similar with both anticoagulants studied. According to our results, flow cytometric analysis of leukocyte surface antigen expressions should be performed using +4°C temperature throughout the process and within 6 hr. *J. Clin. Lab. Anal.* 25:118–125, 2011. © 2011 Wiley-Liss, Inc.

Key words: flow cytometry; lymphocytes; monocytes; neutrophils; CD antigens; sepsis

INTRODUCTION

Recent studies have suggested that the expression of leukocyte surface antigens measured using flow cytometry may serve as diagnostic markers of infection and sepsis and may also predict the outcome of sepsis. The antigens studied most in this context are CD11b and CD64 on neutrophils and monocytes (1–4), but also monocytic human leukocyte antigen (HLA)-DR, CD14, CD40, and CD80 (3,5–7) and CD69 expression on lymphocytes (8) have been of interest.

The methods used in this kind of investigation are, in general, poorly standardized. It is known that several preanalytical and analytical factors (anticoagulant, sample storage and staining temperature, sample storage time, cell separation techniques, erythrocyte lysing, etc.) may have an effect on the expression levels of certain cell surface antigens (e.g., CD11b) (9–11), whereas other antigens (e.g., CD64) (12,13) seem to be quite stable and

unaffected by these factors. Unfortunately, only a few research reports give detailed information on handling of the samples. The comparability of research reports is further complicated by the differences in the presentation of results. The percentage of the antigen-positive cells and unstandardized mean fluorescence intensity have been the two most common ways of reporting the results. Standardized quantitation of cell surface antigen expression by using commercial fluorescent calibration

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beads has been suggested as a way of improving comparability between laboratories and also comparability within laboratories on a day-to-day basis (3,14).

The purpose of this study was to systematically investigate the possible effects of anticoagulant, sample collection, storage and processing temperature, and sample storage time, on the expression of several leukocyte surface antigens. The expression levels of surface antigens were studied by direct immunofluorescence using commercial calibration beads (Quantum molecules of equivalent soluble fluorochrome (MESF) fluorospheres) to quantitate the fluorescence levels. The aim was to determine standard conditions to be used in this type of analysis. The surface antigens of interest were neutrophilic CD11b and CD64, monocytic CD11b, CD14, CD40, CD64, CD80, and HLA-DR, as well as lymphocytic CD69 (separately in CD4+ and CD8+ T cells, B cells, and natural killer (NK) cells).

MATERIALS AND METHODS

The local Ethics Committee of Oulu University Hospital approved the study protocol.

Study Subjects

Our material consisted of five patients (all males, aged 44–68) with severe sepsis requiring intensive care and five healthy staff members (two males and three females, aged 22–62). In this study, the patients and the healthy volunteers were combined into one study population ($n = 10$).

Antibodies

The following monoclonal fluorochrome-conjugated antibodies were used for cell labeling: CD3-PerCP (clone SK7), CD4-FITC (clone SK3), CD8-APC (clone SK1), CD11b-PE (clone D12), CD14-PerCP (clone MQP9), CD16-FITC (clone NKP15), CD19-APC (clone SJ25C1), CD40-FITC (clone 5C3), CD56-FITC (clone NCAM 16.2), CD69-PE (clone L78), CD14-FITC (clone MφP9), CD16-PE (clone B73.1), HLA-DR-PE (clone G46-6), Simultest IgG1/IgG2a-PE (clone X39/X40), and IgG1k-PE (clone X40) from BD Biosciences (San Jose, CA), and CD64-FITC (clone 22(FCψR1)) from Beckman Coulter (Brea, CA).

Blood Samples

Blood was collected into siliconized vacuum tubes containing acid citrate dextrose (ACD) (Venoject, Terumo, Leuven, Belgium) or sodium heparin (BD Vacutainer, Becton Dickinson, Plymouth, UK) as an anticoagulant. Four tubes of blood were collected at the same time: two having ACD and two having heparin as

anticoagulant. One of the ACD and one of the heparin samples were taken into a precooled tube (+4°C) and were immediately transferred back to the ice bath (+4°C), and the other two samples (one ACD and one heparin) were taken and stored at room temperature (RT). Four groups were made: ACD at +4°C, ACD at RT, heparin at +4°C, and heparin at RT. The blood samples were either prepared immediately (within 1 hr of sample collection for flow cytometry) or stored at +4°C (ACD at +4°C and heparin at +4°C) or at RT (ACD at RT and heparin at RT) for 6 hr or 24 hr before staining.

Cell Labeling

50 µl aliquots of total blood containing approximately 0.5×10^6 cells were incubated with the antibodies (3- or 4-color combinations) in the dark at +4°C for 30 min (ACD +4°C and heparin +4°C) or at RT for 15 minutes (ACD RT and heparin RT). All antibodies were used at the concentrations suggested by the manufacturer. After antibody incubation red cells were lysed with FACS lysing solution (BD Biosciences). ACD RT and heparin RT samples were lysed with lysing solution at RT, whereas ACD +4°C and heparin +4°C samples were first lysed with ice-cold lysing solution followed by a second lysing at RT in order to completely eliminate erythrocytes (15). After centrifugation, the cell pellets were resuspended in phosphate-buffered saline and immediately analyzed with the flow cytometer.

Flow Cytometry

FACSCalibur™ flow cytometer and CellQuest software (BD Biosciences) were used for the acquisition and analysis of data. The performance of the flow cytometer was checked regularly by using CaliBRITE-beads (BD Biosciences). The spectral compensations were set up by using stained CompBeads (BD Biosciences). 100,000 total events from each tube were collected except for one patient sample, where only 10,000 total events per tube were collected. The leukocyte subsets were identified based on their forward and side scatter (SSC) characteristics and by using specific monoclonal antibodies. The expressions measured were CD69 expression on CD3+CD4+ and CD3+CD8+ T-lymphocytes, CD19+ B-lymphocytes, and CD3-CD56+ NK cells; CD11b expression on monocytes (CD14+) and neutrophils (CD14-CD16+); CD64 on monocytes (CD14+) and neutrophils (CD14-CD16+); and CD14, CD40, CD80, and HLA-DR on monocytes (CD14+). Interassay standardization and fluorescence quantitation were performed by using Quantum FITC MESF and PE MESF microspheres (Bangs Laboratories, Inc., Fishers, IN) according to the manufacturer's instructions. The

scattergrams shown in the figures were produced using BD FACSDiva Software (BD Biosciences).

Data Analysis

The possible differences in antigen fluorescence intensities expressed in MESF between the time points, between the two anticoagulants, and between the two temperatures were investigated using PASW statistics 18 (IBM, Chicago, IL) and either Friedman's test or Wilcoxon's signed rank test where appropriate.

RESULTS

Influence of Temperature

In this study, the samples were collected, stored, and stained either at +4°C or at RT. At the first analysis point (within 1 hr of sample collection), the fluorescence intensity of nearly all of the antigens studied was higher among samples maintained at RT than at +4°C (Figs. 1–3). This effect was almost identical with both of the anticoagulants, ACD and heparin.

Specifically, with ACD-anticoagulated samples, the fluorescence intensity of all other antigens except for CD69 antigen on CD4+ T cells and NK cells and CD80 antigen on monocytes was higher at RT than at +4°C even after only 1 hr of storage (Figs. 1–3; Wilcoxon's test $P < 0.05$, data not shown). For CD69 on NK cells and CD80 on monocytes, the statistical difference between the temperatures occurred after 6 hr of storage ($P < 0.05$) and for CD69 on CD4+ T cells after 24 hr ($P < 0.05$). In heparin-anticoagulated samples, there was a statistical difference between the temperatures after 1 hr for all of the antigens ($P < 0.05$, data not shown).

The effect of temperature at the 1 hr analysis point was most prominent for CD11b (for both monocytes and neutrophils), monocytic CD14 and especially monocytic HLA-DR antigens.

Influence of Storage

During storage of the samples at +4°C, there was a decrease in fluorescence intensity of the monocytic antigen CD64 (Fig. 2). This was already observed with both of the anticoagulants after 6 hr of storage

Fig. 1. CD69 antigen fluorescence intensity on lymphocytes of ACD- and heparin-anticoagulated samples during storage at +4°C or at RT for 1, 6, and 24 hr. The results are presented as median fluorescence intensities of ten patient and control samples in MESF. Lymphocyte subtypes are presented separately: CD4+ T cells (A), CD8+ T cells (B), NK cells (C), and B cells (D), and the y-axis scale varies depending on the cell population. *P*-values were obtained from Friedman's test for related samples and indicate a change of the fluorescence intensity within the samples of individuals during 24 hr storage (*p*(str)).

Fig. 2. CD11b antigen fluorescence intensity on monocytes (A) and neutrophils (B) and CD64 antigen fluorescence intensity on monocytes (C) and neutrophils (D) of ACD- and heparin-anticoagulated samples during storage at +4°C or at RT for 1, 6, and 24 hr. The results are presented as median fluorescence intensities of ten patient and control samples in MESF. The y-axis scale varies depending on the antigen and cell population. *P*-values were obtained from Friedman's test for related samples and indicate a change of the fluorescence intensity within the samples of individuals during 24 hr storage (*p*(str)).

(Wilcoxon's test, $P < 0.05$, data not shown). For all other antigens, no marked changes in the fluorescence intensities were noted even at 24 hr (Figs. 1–3). Only in a few single patients' samples was a change in some antigens seen after 24 hr.

Conversely, the storage of the samples for 24 hr at RT caused increased fluorescence intensity of all of the antigens (Friedman's test $P < 0.05$; Figs. 1–3). With most of the antigens, statistically significant changes in the intensity were already noticed after 6 hr of storage at RT (data not shown). On CD11b and HLA-DR antigens up to between five- and ten-fold increases in the fluorescence intensities were seen during 24 hr of storage. Changes seen on the fluorescence intensities of monocytic CD64 antigen and antigen CD69 of some lymphocyte subtypes during 24 hr of storage at RT were minor.

Influence of Anticoagulation

The main trends were similar with both of the anticoagulants: fluorescence intensities were higher at RT than at +4°C and an increase of fluorescence intensity was noticed during storage of the samples at

RT (Figs. 1–3). At +4°C, there were no clear differences in fluorescence intensities of any of the antigens studied in fluorescence intensities of any of the antigens studied at any of the time points studied. At RT, the 1 hr fluorescence intensities were quite similar with both of the anticoagulants, but, for all of the antigens, the increase in the fluorescence intensity seen at RT was higher in heparin samples than in ACD-anticoagulated samples.

Another finding was that, in some samples with increased cell debris, it was easier to define margins of some cell populations in ACD samples than in heparin samples (Fig. 4).

An example of scattergrams showing the effects of temperature, storage, and anticoagulation on CD11b fluorescence intensity of monocytes is shown in Figure 5.

DISCUSSION

In this study, the effects of temperature, storage, and anticoagulation on the expression levels of a wide variety of leukocyte surface antigens were investigated using standardized quantitative flow cytometry. There are two main findings in the study: first, handling of

Fig. 3. HLA-DR (A), CD14 (B), CD40 (C), and CD80 (D) antigen fluorescence intensity on monocytes of ACD- and heparin-anticoagulated samples during storage at +4°C or at RT for 1, 6, and 24 hr. The results are presented as median fluorescence intensities of ten patient and control samples in MESF. The y-axis scale varies depending on the antigen. *P*-values were obtained from Friedman's test for related samples and indicate a change of the fluorescence intensity within the samples of individuals during 24 hr storage (*p*(str)).

samples at RT results in higher fluorescence intensity compared with handling at +4°C; second, fluorescence intensity changes during a 6 or 24 hr storage of the samples, more so at RT but also at +4°C.

Several preanalytical and analytical factors are known to affect the expression levels of certain cell surface antigens when studied by flow cytometric immunophenotyping (10,11,16,17). When applying this kind of method to clinical practice, one should be aware of the possible inducing or masking effects that the preanalytical and analytical factors might have on the antigen expressions studied. There are some contradictory reports concerning, for example, the efficiency of CD11b to differentiate septic patients from nonseptic patients (18–21) or the efficiency of HLA-DR or CD14 to predict the outcome of septic patients (5,6,22–25). One may wonder whether these differences might be explained, at least in part, by the differences in preanalytical factors in these studies (5).

In this study, in order to avoid *in vitro* activation of leukocytes, the manipulation of the samples before staining of the surface antigens was kept as minimal as possible. Therefore, direct immunofluorescence and

whole blood procedure with red cell lysing after the staining was chosen. Further, in order to increase the comparability of the results from day-to-day we quantitated the expression levels of surface antigens by using commercial calibration beads (Quantum MESF fluorospheres).

For most of the antigens studied here no systematic evaluation of the effects of temperature and storage has hitherto been performed, and, in the literature, for all of the antigens both room and cold preparation temperatures have been used. In this study, the fluorescence intensity of CD11b both on neutrophils and monocytes at RT rose rapidly, which is in line with previously published results (10,11,16,21). We also saw a major increase in the intensities of HLA-DR and CD14 on monocytes within 6 hr when the samples were handled at RT. This kind of result has not been previously published. In line with our results, Kylanpää-Back et al. (26) and Aalto et al. (22) did not observe changes in the HLA-DR or CD14 staining properties of monocytes during 24 hr storage at 0°C, respectively. According to the literature CD64 antigen is stable and largely unaffected by preanalytical variables (12–14).

Fig. 4. In some heparin samples it was more difficult to define the margins of some cell populations compared with ACD samples. This is illustrated in the example of CD16 positive neutrophils gated on dark gray in ACD-anticoagulated samples (A and B) and heparin-anticoagulated samples (C and D) stored for 6 hr at RT.

However, in this study, an increase in the fluorescence intensity of neutrophilic CD64 antigen was observed at RT during storage of 24 hr. Interestingly, at $+4^{\circ}\text{C}$ the fluorescence intensity of monocytic CD64 antigen slightly decreased during storage. To our knowledge, the effects of temperature and storage on the expression levels of CD40, CD80, and CD69 antigens have not been previously evaluated at all.

A common anticoagulant, ethylenediaminetetraacetic acid, was excluded from this study as it is known to partly inhibit the binding of the CD11b antibody used in this experiment to its epitope (11,27). According to the literature, both ACD and heparin anticoagulants have been and probably can be used in leukocyte activation studies, although one should be aware that the possible effects of an anticoagulant may be antigen-dependent (9). If the samples are stored at RT, heparin may preserve the cell integrity for a longer time but during the first 24 hr no major differences have been observed. In our study, no major differences between anticoagulants were observed at $+4^{\circ}\text{C}$, but at RT heparin tended to produce higher fluorescence, especially at 24 hr. We would prefer ACD as an anticoagulant, as in some samples with increased cell debris, it was easier to define margins of some cell populations with ACD samples than with heparin samples (Fig. 4).

The information presented by this study is only methodological: a blood sample of a single test person ends up in different fluorescence intensities depending on the handling of the sample. This should be taken into consideration when investigating leukocyte surface molecules and interpreting subsequent results. In particular, results reported after storage of samples for more than 6 hr at RT should be interpreted cautiously. In order to obtain wider ranges of fluorescence intensities for the study, we included samples from both sepsis patients and healthy individuals in the study. However, due to the relatively small study population ($n = 10$) and the heterogeneity of the patients included, the fluorescence intensities between the patient samples and the healthy control samples were not compared. Because of the methodological approach and the high cost of the reagents as well as the wide range of antigens investigated, we did not consider it necessary to analyze more patients and controls merely for statistical comparison. Moreover, it is important to emphasize that the effects of temperature, anticoagulation, and storage were similar in both patients and controls.

In conclusion, this study shows in a quantitative way that storing the blood samples at RT may have pronounced effects on the intensities of several leukocyte surface antigens, something which is of interest in

Fig. 5. Typical scattergrams of the effects of temperature, storage, and anticoagulation on CD11b fluorescence intensity of CD14-positive monocytes. The samples were taken simultaneously from the same patient: ACD-anticoagulated samples stored at +4°C for 1 hr (A) and 24 hr (B), and similarly at RT for 1 hr (C) and 24 hr (D), as well as heparin-anticoagulated sample stored at +4°C for 1 hr (E) and 24 hr (F), and similarly at RT for 1 hr (G) and 24 hr (H). The numerical values shown in figures represent median fluorescences of CD11b in monocytes (FI_{med}) and corresponding values in MESF.

leukocyte activation studies (e.g., sepsis). At +4°C the changes during storage are lower. According to our results it would be recommended that blood samples for flow cytometric leukocyte measurements should be collected, prepared, and stored at +4°C, and their

analysis should be performed as soon as possible, but at most within 6 hr.

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