The sample processing time interval as an influential factor in flow cytometry analysis of lymphocyte subsets

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The objective of this paper is to propose a protocol to analyze blood samples in yellow fever 17DD vaccinated which developed serious adverse events. We investigated whether or not the time between sample collection and sample processing could interfere in lymphocyte subset percentage, for it is often impossible to analyze blood samples immediately after collection due to transport delay from collection places to the flow cytometry facility.

$CD4^+CD38^+$ T, $CD8^+CD38^+$ T, $CD3^+$ T, $CD19^+$ B lymphocyte subsets were analyzed by flow cytometry in nine healthy volunteers immediately after blood collection and after intervals of 24 and 48 h. The whole blood lysis method and gradient sedimentation by Histopaque were applied to isolate peripheral blood mononuclear cells for flow cytometry analyses. With the lysis method, there was no significant change in lymphocyte subset percentage between the two time intervals (24 and 48 h). In contrast, when blood samples were processed by Histopaque gradient sedimentation, time intervals for sample processing influenced the percentage in T lymphocyte subsets but not in B cells. From the results obtained, we could conclude that the whole blood lysis method is more appropriate than gradient sedimentation by Histopaque for immunophenotyping of blood samples collected after serious adverse events, due to less variation in the lymphocyte subset levels with respect to the time factor.

Key words: yellow fever - flow cytometry - lymphocyte subsets

Yellow fever (YF) remains a serious health problem in endemic areas of tropical and subtropical Africa and South America (Vasconcelos 2003). Approximately 90% of the infected people have mild infections or are asymptomatic, while 10% develop severe morbidness leading to 50% case-fatality. In Brazil, this illness can be prevented through YF 17DD virus substrain vaccine, which induces protective neutralizing antibodies of long duration.

Although the YF 17DD substrain is one of the most successful vaccines ever elaborated, rare cases of serious postvaccinal disorders have been recorded (Vasconcelos et al. 2001). The virus isolates from two post YF 17DD vaccination fatal cases demonstrated genetic stability and attenuated phenotype, suggesting that some immune defense peculiarities of the host might have been responsible for such adverse events (Galler et al. 2001). To monitor such serious adverse events accompanying the YF vaccine, Fiocruz-Biomanguinhos has created a task force, which together with the Health Ministry will be responsible for immunological profile analyses and detection of YF virus as well as any other pathogenic agent that may quite possibly be related to clinical complications presented by the patient, e.g. leptospirosis.

With the objective of defining a protocol to analyze blood samples from patients with serious adverse events after YF 17DD vaccination, when the procedure cannot be effected immediately due to sample shipping delays, we have initially studied the influence of the time interval on the lymphocyte subset profiles by flow cytometry using the whole blood lysis method (Loken et al. 1988) and Histopaque gradient sedimentation (Noble et al. 1968). The flow cytometry technique has been successfully employed to enumerate and identify lymphocyte specific sets or subsets from blood specimens after YF vaccination (Santos et al. 2005, Co et al. 2002). $CD4^+CD38^+$ T, $CD8^+CD38^+$ T, $CD3^+$ T, $CD19^+$ B lymphocyte subsets were analyzed in nine healthy volunteer vaccinees immediately after blood collection (control) and after intervals of 24 and 48 h, following the previous recommended procedures (Mandy et al. 2003, Gratama 2006). All samples were collected in 0.105 M sodium citrate anticoagulant (BD Vacutainer Systems) and stored at room temperature with protection from light. Temperatures above 37ºC or below 4ºC might cause cellular destruction and affect flow cytometry measurements (Mandy et al. 2003)

Dual and triple-staining labeled monoclonal antibodies [CD3 (IgG1)-Fluorescein Isothiocyanate (FITC)/ CD19-Phycoerythrin (PE); CD8-PE/CD38-FITC/CD4-Phycoerythrin-Cyanine 5 (PC5)] and IgG1-FITC/IgG1-PE isotype control (Immunotech, Beckmann Coulter, Marseille, France) were used. The stained cells were run in an EPICS ALTRA flow cytometer (Beckmann Coulter, Hialeah, FL, US) equipped with an argon ion laser. Ten

thousand events were obtained and analyzed using the Expo 32 software (Beckmann Coulter). The forward scatter and side scatter profiles distinguished lymphocytes and monocytes. An electronic gate was created around lymphocytes. A multifactor analysis of variance (ANOVA) followed by the least significant difference (LSD) procedure, Statgraphics Plus Version 4.1 Software (US), was applied to evaluate statistically significant differences between means. With this method, there is a 5% risk of identifying each pair of means significantly different when the actual difference equals 0. Differences were considered as statistically significant at $p \leq 0.05$.

The results obtained demonstrated that, when the whole blood lysis method was utilized for both time intervals, a significant increase of cellular debris was detected. However this fact did not hamper the detection of lymphocyte subsets using labeled monoclonal antibodies. There was also no significant change in lymphocyte subset percentage during the two time intervals analyzed (CD3^{+} T cells $p = 0.6720$; CD19^{+} B cells $p =$ 0.1112 (Figs 1A, 2A); CD4^{+} T cells $p = 0.861$; CD4^{+}CD38^{+} T cells $p = 0.7467$; CD8^{+} T cells $p = 0.2245$; CD8^{+}CD38^{+} T cells $p = 0.1166$ (Figs 1A, 2B). In contrast, the time interference in lymphocyte subset percentage could be observed when blood samples were processed by Histopaque gradient sedimentation, which resulted in a significant difference from 24 to 48 h post collection, except for B cells (CD3^{+}T cells $p < 0.0001$; CD19^{+}B cells $p = 0.1811$ (Figs 1B, 2A); CD4^{+} T cells $p = 0.0001$; CD4^{+}CD38^{+} T cells $p = 0.0001$; CD8^{+} T cells $p < 0.0001$; CD8^{+}CD38^{+} T cells $p < 0.0001$ (Figs 1B, 2B).

The YF 17DD vaccine, produced in Fiocruz-Biomanguinhos, Brazil, has proved to be a safe and efficient vaccine, providing suitable results regarding neutralizing antibody production (Marchevsky et al. 2003). However, some serious adverse events after vaccination have been reported (Galler et al. 2001, Vasconcelos et al. 2001), supporting the idea of other authors that cellular immune response peculiarities of the host might induce adverse events after YF vaccination (Martin et al. 2001).
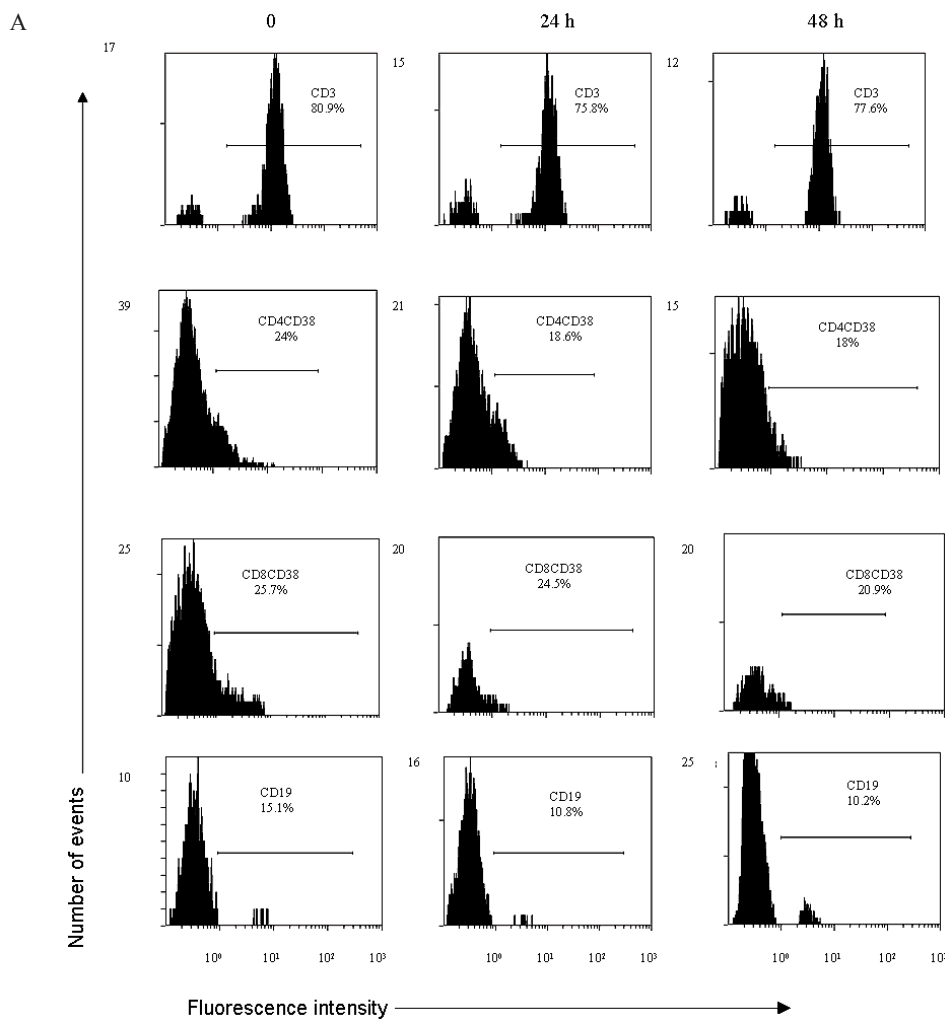

Fig. 1A: phenotypic analyses of the lymphocyte subsets after dual and triple-stained with CD3-FITC/CD19-PE and CD4-PC5/CD8-PE/CD38-FITC monoclonal antibodies. The histograms represent the percentage of CD3^{+} T cells, CD4^{+}CD38^{+} T cells, CD8^{+} CD38^{+} T cells, and CD19^{+} B cells analyzed immediately after blood collection (0), 24 and 48 h later. These samples were processed by whole blood lysis method.

In this study, the flow cytometry method was chosen to monitor T and B lymphocyte subsets, because it is an efficient tool for definition and quantification of lymphocytes. The typing of lymphocyte subsets may be influenced by a variety of technical specificities, such as sample and lymphocyte preparations, time and storage temperature (Ekong et al. 1993).

We observed in forward and side scatter dot plot lymphocyte subsets at 24 and 48 h after collection an area of lower definition than that of the control sample with both techniques. The gradient sedimentation by Histopaque was not as efficient as the whole blood lysis method when samples were analyzed 48 h post-collection.

The results displayed no change in the relative levels of T and B cells when whole blood lysis was analyzed at 48 h post-collection, but a significant change in T lymphocyte subsets as exhibited when these cells were separated by a density gradient. This fact may be due to a weak definition between peripheral blood mononuclear cells and non-nucleated red cell and debries in the gra-

dient sedimentation, which can lead to a technical error in the lymphocyte separation from red cell and granulocyte contamination.

In a previous study, Nicholson et al. (1984) reported similar results when they analyzed T cells in PBMC preparations 24 h after collection. When PBMC was isolated in a density gradient, these authors observed a change in lymphocyte subset levels in whole blood samples that were stored at room temperature for 24 h with a significant increase in T cells and decrease in B cells. In contrast, we witnessed no significant change in B and T cell levels when whole blood samples were stored for 48 h. It is possible that the discrepancies between our findings and theirs are associated with contamination of cellular elements, e.g. neutrophils, nonnucleated red cells, that may take place in blood sample not manipulated immediately after collection.

The data presented herein indicate that when the flow cytometric analysis cannot be carried out immediately, the lysis whole blood method should be adopted to analyze lymphocyte subsets from patients with serious ad-
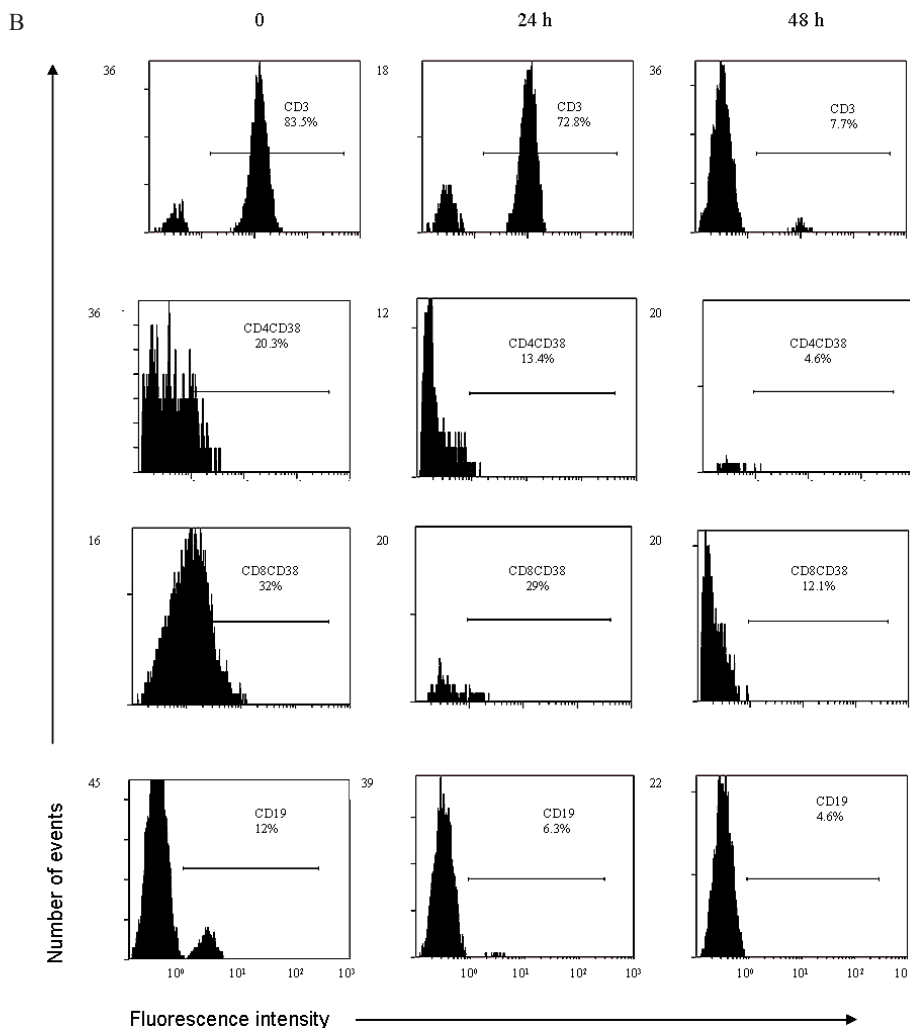

Fig. 1B: phenotypic analyses of the lymphocyte subsets, isolated in a gradient sedimentation by Histopaque, after dual and triple-stained with CD3-FITC/CD19-PE and CD4-PC5/CD8-PE/CD38-FITC monoclonal antibodies. The histograms represent the percentage of CD3^{+} T cells, CD4^{+}CD38^{+} T cells, CD8^{+} CD38^{+} T cells, and CD19^{+} B cells analyzed immediately after blood collection (0), 24 and 48 h later.
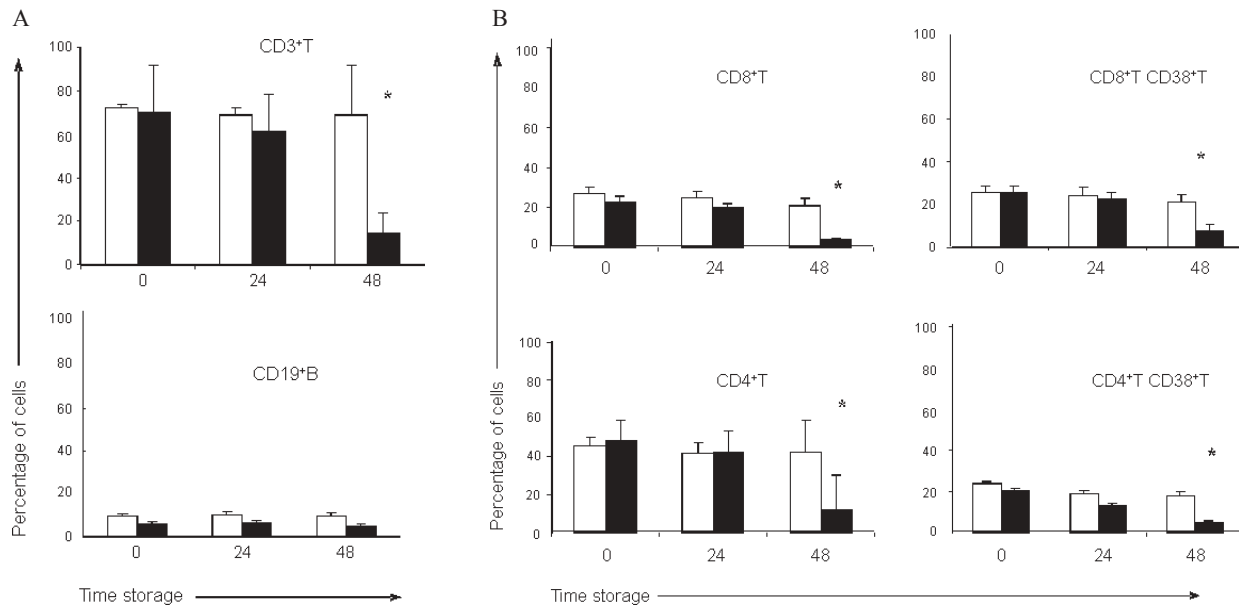
A

B

Fig. 2A: the average of CD3$^+$ T and CD19$^+$ B cell percentages from peripheral blood of nine healthy volunteers. The bars show the mean of percentage of the lymphocyte subsets analyzed on flow cytometer, immediately after blood collection (control, 0), 24, and 48 h later. The black bars represent gradient sedimentation by Histopaque, and the white bars represent the whole blood lysis method. * indicates statistically different results between whole blood lysis method and gradient sedimentation by Histopaque. Standard error ($\pm$ SE) $p < 0.05$. Fig. 2B: CD8$^+$ T, CD8$^+$CD38$^+$ T, CD4$^+$ T, CD4$^+$CD38$^+$ T cells percentages from PBMC from peripheral blood of nine healthy volunteers. The bars show the mean of percentage of the lymphocyte subsets analyzed on flow cytometer, immediately after blood collection (control, 0), 24, and 48 h later. The black bars represent gradient sedimentation by Histopaque, and the white bars represent the whole blood lysis method. * indicates statistically different results between whole blood lysis method and gradient sedimentation by Histopaque. Standard error ($\pm$ SE) $p < 0.05$.

verse events after 17DD vaccination. This method can reduce variation over time on lymphocyte subset levels in the immunophenotyping, approximating results obtained with samples analyzed immediately after collection.

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