THE JOURNAL OF PREVENTIVE MEDICINE
2003; 11 (1): 67-74

FLOW-CYTOMETRIC ANALYSIS OF SPECIFIC-PROLIFERATION IN TUBERCULOSIS USING THE CFSE DYE DILUTION TECHNIQUE

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Abstract. In the present study we evaluated the *in vitro* lymphocyte specific blastogenesis in response to stimulation with PPD (purified-proteine-derivate) in tuberculosis patients using the flow-cytometry method based on carbofluorescein diacetate succinimydil ester (CFSE) dye dilution technique. 14 individuals (7 PPD+ healthy donors and 7 tuberculosis patients) were studied. Mononuclear cells from peripheral blood or pleural fluid were initially stained with 1.25 μM CFSE, then they were cultivated with or without 20 μg/ml PPD for up to 7 days. At indicated time points the cells were collected, stained with anti-CD3 Cy Chrome and analyzed on a FACSCalibur flow cytometer. The results showed a poor reactivity of peripheral lymphocytes from tuberculosis patients as compared to that recorded in healthy, PPD-reactive persons, and to the values observed in the proliferative response of cells from pleural effusion.
Key words: CFSE; tuberculosis; lymphocyte proliferation.

Rezumat. În acest studiu a fost investigată capacitatea de proliferare a limfocitelor stimulate cu PPD, la pacienţii cu tuberculoză, prin analiza flow-citometrică a celulelor marcate cu carbofluorescein diacetate succinimydil ester (CFSE). Au fost investigate 14 persoane: 7 pacienţi cu tuberculoza şi 7 persoane sănătoase, PPD-pozitive. Celule mononucleare din sânge periferic sau lichid pleural au fost iniţial marcate cu 1.25 μM CFSE şi apoi cultivate cu şi fără 20 μg/ml PPD timp de 7 zile. La anumite intervale de timp celulele au fost colectate, marcate cu anti-CD3 Cy Chrome şi analizate flow-citometric. Rezultatele obţinute au indicat o slabă capacitate de proliferare a limfocitelor periferice la pacienţii cu tuberculoză în comparaţie cu celulele obţinute din lichidul pleural si cu valorile obţinute în cazul persoanelor sănătoase PPD-reactive.
Cuvinte cheie: CFSE; tuberculoză; proliferare limfocitară.

INTRODUCTION

The lymphocyte transformation test is one of the common assays used in immunological studies in order to assess the immune responsiveness to various stimuli. The cell proliferation is commonly assessed by measuring the tritiated thymidine incorporation (^{3}H-TdR) (1). However, the use of radioisotopes as a component of this method determined the development of other less hazardous alternatives.

One of the latest solutions is the flow cytometric analysis of cell division using the dilution of carbofluorescein diacetate succinimydil ester (CFSE) (2). Identified by Weston and Parish in 1990 as a long-term tracking dye for lymphocytes migration studies (3), CFSE is a non-fluorescent molecule which consists of two acetate side chains and a succinimidyl ester group. It becomes fluorescent only after entering the cells and the acetate groups are displaced by endogenous esterases (4). The succinimidyl ester binds covalently to cytoplasmic free amine groups without affecting cellular function. During cell division CFSE is equally distributed between progeny (2) so that the fluorscence of the cells stained with CFSE at the beginning of the culture will decrease as the cells proliferate.

Since its discovery, the CFSE dye dilution technique has been used in a variety of immunological studies such as investigation of phenotypic changes associated with T cell effector/ memory function (5), the B cell isotype switching during division (6), the blastogenic response to *Candida* (7) or the T cell cytokine production related to the process of division (8,9).

In the present study we evaluate the *in vitro* lymphocyte specific blastogenesis in response to stimulation with PPD in tuberculosis patients using the flow cytometry method based on CFSE.

MATERIAL AND METHODS

Subjects

14 individuals aged between 20 and 45 years were studied: 7 positive PPD healthy donors and 7 tuberculosis patients. The intradermal test was performed with 2 units of tuberculin (Cantacuzino Institute, Bucharest) and the diagnosis of tuberculosis was established after considering the clinical status, the chest X-ray and the positive sputum culture. Patients were studied before any antituberculostatic treatment was initiated. One of the patients was diagnosed with TB pleurisy, so that his exudative pleural fluid collected at the beginning of the specific treatment was also investigated for lymphocyte blastogenesis.

CFSE labeling and cell culture

40 ml of venous blood or pleural fluid was collected in heparinised syringe and processed for mononuclear cells isolation by density sedimentation over Ficoll-Hystopaque (Sigma). The exudative fluid was first centrifuged; then the resuspended pellet was layered on Ficoll. Trypan blue staining was used to check the cell viability which was always over 95%.

Prior to culture, the mononuclear cells were labeled with CFSE (Molecular Probes, USA) using a slightly modified technique, originally devised by Lyons et al (4). Briefly, 1 x 10^7 cells/ml were incubated with 1.25 μM CFSE for 10 min at 37^0C. The labeling was quenched by adding one volume of cold autologous plasma and the cells were washed twice in cold RPMI 1640 (Sigma).

According to previous pilot experiments, 10^6 cells/ml were cultured in the presence or absence of antigen in 24-well plates (Nunc, Denmark) at 24^0C, humid atmosphere, 5% CO_2 in RPMI 1640 supplemented with antibiotics and 5% autologous plasma. The cells

were stimulated for up to 7 days with 20 μg/ml PPD free of preservatives, or 5 μg/ml phytohemaglutinin (PHA) which served as a positive control for cell reactivity.

Flow cytometry analysis

At indicated time points, the cells were collected, stained with anti-CD3 Cy Chrome (Pharmingen) in the dark, at room temperature for 30 minutes, and the analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson) using the CellQuest software. 20,000 events per sample were acquired and the viable lymphocytes were selected according to their forward (FSC) and side scatter (SSC) characteristics. The CD3+ cells were gated on a SSC versus Fl3 (Cy Chrome) dot-plot then a CFSE fluorescence of the CD3+ lymphocytes was displayed as a histogram which allowed us to establish the proportion of the specific-proliferated population.

RESULTS AND DISCUSSION

There are studies which investigated the proliferative responses of peripheral blood mononuclear cells (PBMCs) in *M. tuberculosis* infection by determination of the tritiated thymidine or bromo-deoxyuridine incorporation (10,11,12, 13,14).

These commonly used assays have some limits because they measure only the division at a population level while the CFSE dye dilution technique allows us to track proliferation in subsets of cells.

This study is the first report concerning the specific blast transformation in tuberculosis by flow cytometry analysis using the CFSE dilution technique.

Thus, peripheral mononuclear cells from tuberculosis patients or healthy individuals were first labeled with 1.25 μM CFSE then cultured with antigen (20 μg/ml PPD) for 7 days. At days 0, 5 and 7 the cells were harvested, stained with anti CD3 Cy-Chrome and analyzed by flow-cytometry.

As a positive control of the cells reactivity, PBMCs from the same persons were incubated in the presence of a policlonal stimulus (5μg/ml PHA) and were analyzed on days 0, 3 and 5, as it is known that mitogen stimulation requires less time of incubation than the antigens.

A representative example of analysis is illustrated by figures 1 and 2. All viable lymphocytes, the small ones together with the blasts, which are defined as having greater forward and light scatter, were gated on a FSC/SSC dot-plot (left panels). As the lymphocytes progress through the division process they increase in size and their CFSE fluorescence intensity diminishes (middle panels). The CD3+ cells were selected by gating them on the Fl3/SSC dot-plot and the proportion of the T cells which underwent the blastic transformation were determined on histograms of CFSE fluorescence intensity (right panels).

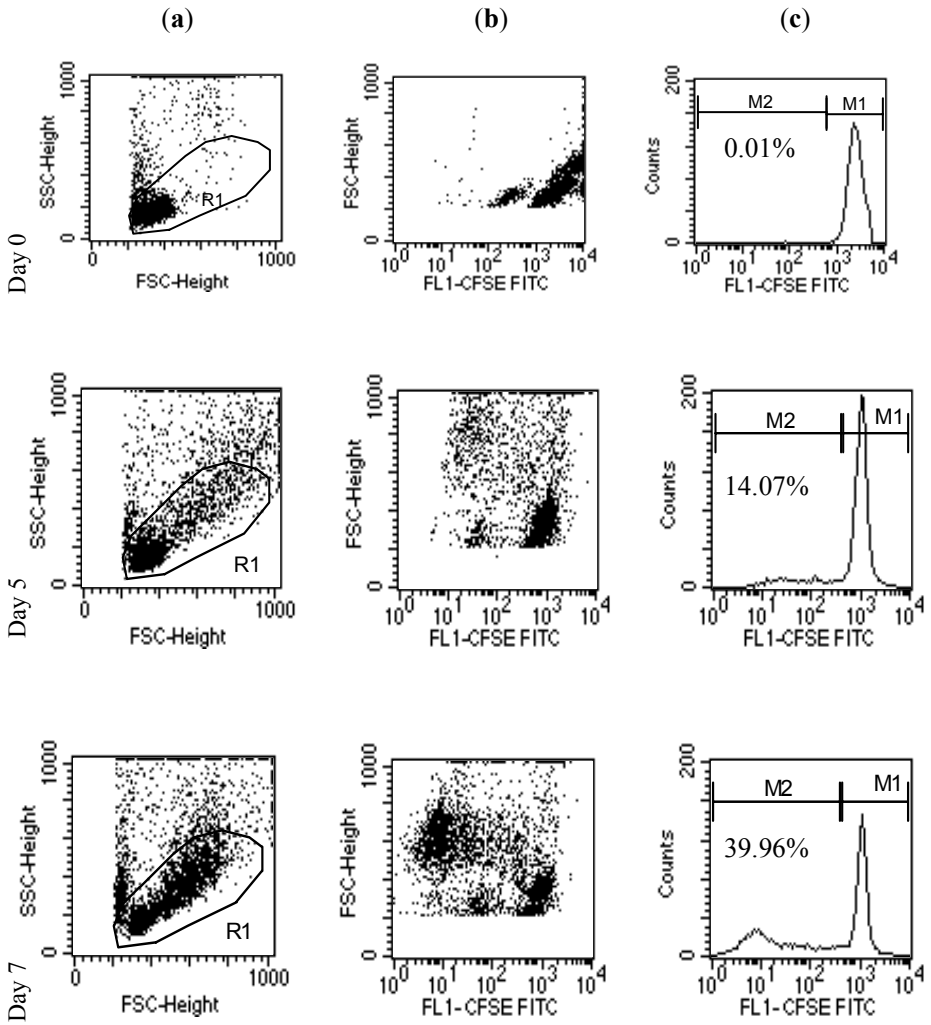

Fig. 1 Flow-cytometry analyses of the lymphocyte proliferation in a healthy, PPD-reactive, subject

PBMCs were labeled with CFSE prior to culture and stimulated for up to 7 days with 20μg/ml PPD. At the indicated time points, the cells were collected, stained with anti CD3 Cy-Chrome and analysed on a FACSCalibur flow-cytometer. (**a**) Viable small and blast lymphocytes selected on the basis of their size (FSC) and granularity (SSC) characteristics. (**b**) Forward scatter as a measure of cell size on the vertical axes, and CFSE fluorescence on the horizontal axes. As the cells proliferate they increase in size and decrease in CFSE intensity. (**c**) Fluorescence histograms of gated CD3+ lymphocytes. Percentages represent the proliferated T lymphocytes from the analyzed population.

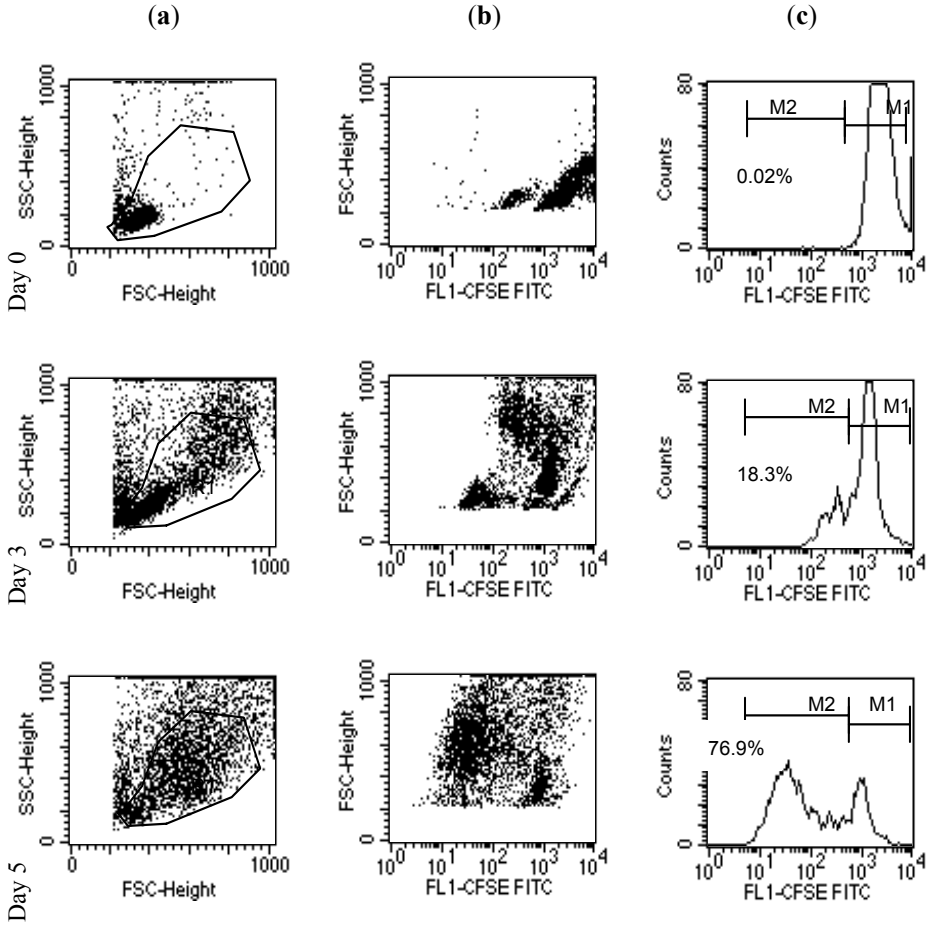

Fig. 2 Flow-cytometry analysis of the lymphocyte proliferation in a healthy subject

PBMCs were labeled with CFSE prior to culture and stimulated for up to 5 days with 5 µg/ml PHA. At the indicated time points, the cells were collected, stained with anti CD3 Cy-Chrome and analysed on a FACSCalibur flow-cytometer. (**a**) Viable small and blast lymphocytes selected on the basis of their size (FSC) and granularity (SSC) characteristics. (**b**) Forward scatter as a measure of cell size on the vertical axes, and CFSE fluorescence on the horizontal axes. As the cells proliferate they increase in size and decrease in CFSE intensity. (**c**) Fluorescence histograms of gated CD3+ lymphocytes. Percentages represent the proliferated T lymphocytes from the analyzed population.

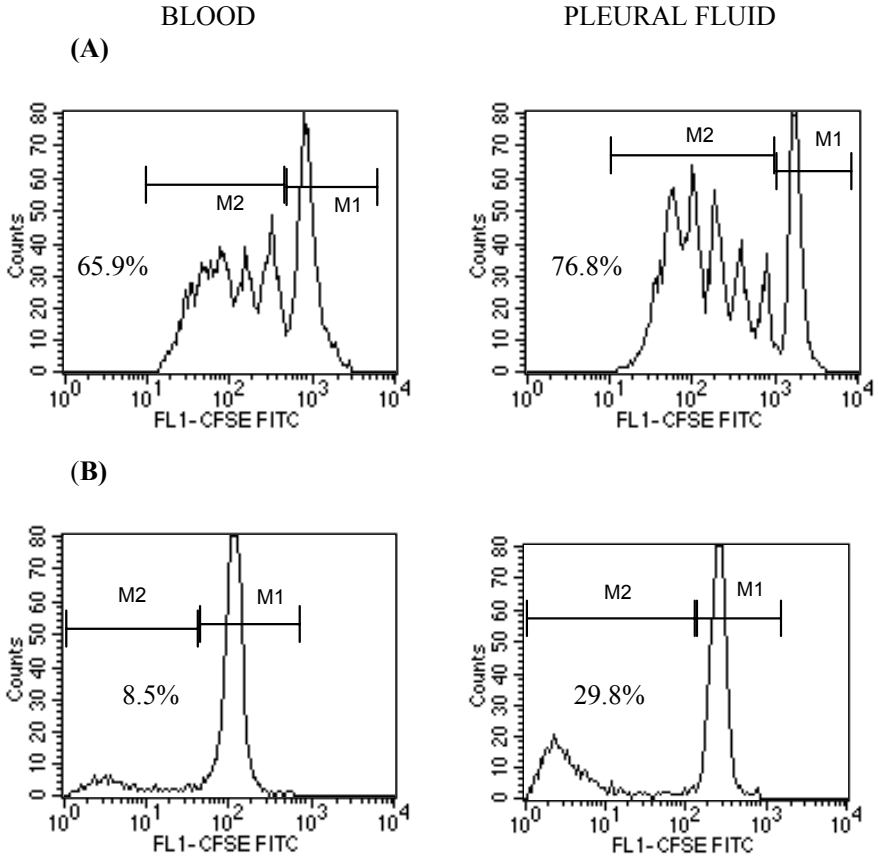

Fig. 3 Comparative study of the T-cells proliferation in blood and pleural fluid in a patient with tuberculosis pleurisy

Mononuclear cells were labeled with CFSE prior to culture and incubated for 5 days with 5 μg/ml PHA (**A**) and for 7 days with 20 μg/ml PPD (**B**). Gated CD3+ lymphocytes are displayed on CFSE fluorescence histograms to demonstrate the decrease of fluorescence intensity during divisions. Figures represents the percentage of $CFSE^{low}CD3+$ cells (proliferated cohort) from the analyzed population.

The results indicated that even if the lymphocytes function was preserved, as demonstrated by stimulation with PHA (data not shown), the PPD-specific T cell proliferation was minimal in the TB patients group as compared with the levels recorded in the healthy subjects (table 1).

Table1. Percentages of proliferated CD3+ peripheral lymphocytes on day 7 of cultures stimulated with PPD in tuberculosis patients and healthy subjects

CD3+ lymphocytes (%)	
TB	**HEALTHY**
7.2	35.9
11.5	39.9
8.5	30.4
10.0	29.8
5.7	33.5
9.2	31.8
6.7	30.8

But, when the T lymphocytes from the TB pleural exudate were studied a significant T cell proliferation occurred in response to PPD stimulation (fig. 3).
These data seem to be in agreement with those studies which suggest that the immunosuppression observed in peripheral blood lymphocytes during active tuberculosis may be related to the "sequestration" of the specific T cell subset at the site of the inflammation (15,16,17,18).
If this is the case at the beginning of the disease we can assume that at the end of the specific chemotherapy the peripheral T-cells may recover their potential to expand in response to PPD stimulation.

Activation of T lymphocytes which in turn activate macrophages is a prerequisite phenomenon to restrict the growth of *M. tuberculosis* and thereby to control the infection (19).

CONCLUSIONS

- The flow-cytometry method employed in order to assess PPD-specific proliferative response of peripheral blood lymphocytes labeled with CFSE can be considered as a very useful technique which allows a precise monitoring of the efficiency of the treatment in tuberculosis patients.
- Moreover, its use in high-risk groups testing, such as hospital personnel, would permit the detection of subclinical tuberculosis infection and the administration of prophylactic chemotherapy thus reducing the potential for disease transmission**.**

ACKNOWLEDGEMENTS

We express our gratitude to Dr. Henriette Stavri from the Cantacuzino Institute of Bucharest who kindly provided us the PPD solution.

REFERENCES

1. Maghni K, Nicolescu OM, Martin JG: *Suitability of cell metabolic colorimetric assays for assessment of CD4+ T cell proliferation: comparison to 5-bromo-2-deoxyuridine (BrdU) ELISA*. 1999, 223: 185-194.
2. Lyons AB, Hasbold J, Hodgkin PD: *Flow cytometric Analysis of cell division History Using Dilution of Carboxyfuorescein Diacetate Succinimidyl Ester, a Stably Integrated Fluorescent Probe*. Methods in Cell Biology, 2001, 63: 375-397.

3. Parish CR: *Fluorescent dyes for lymphocyte migration and proliferation studies*. Immunol. Cell Biology, 1999, 77: 499-508.
4. Lyons AB: *Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution*. J. Immunol. Methods, 2000, 243: 147-154.
5. Lee WT, Pelletier WJ: *Visualizing Memory Phenotype Development after in Vitro Stimulation of CD4+ T Cells.* Cellular Immunology, 1998, 188; 1-11.
6. Hasbold J, Lyons AB, Kehry MR, Hodgkin PD: *Cell division number regulates IgG1 and IgE switching of B cells following stimulation by CD40 ligand and IL-4*. Eur. J. Immunol., 1998, 28: 1040-1051.
7. Angulo R, Fulcher DA: *Measurement of Candida-Specific Blastogenesis: Comparison of Carbofluorescein Succinimidyl Ester Labelling of T Cells, Thymidine Incorporation, and CD69 Expression*. Cytometry, 1998, 34: 143-151.
8. Gett AV, Hodgkin PD: *Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation*. Proc. Natl Acad. Sci. USA, 1998, 95: 9488-9493.
9. Bird JJ, Brown DR, Mullen AC et al.: *Helper T cell differentiation is controlled by the cell cycle*. Immunity, 1998, 9: 229-937.
10. Rosenkrands I, Weldingh K, Ravn P et al.: *Differential T-Cell Recognition of Naïve and Recombinant Mycobacterium tuberculosis GroES*. Infect. Immun., 1999, 67: 5552-5558.
11. Delgado JC, Tsai EY, Thim S et al: *Antigen-specific and persistent tuberculin anergy in a cohort of pulmonary tuberculosis patients from rural Cambodia*. PNAS, 2002, 99: 7576-7581.
12. Demissie A, Ravn P, Olobo J et al.: *T-Cell Recognition of Mycobacterium tuberculosis Culture Filtrate Fractions in Tuberculosis Patients and Their Household Contacts*. Infect. Immun., 1999, 67: 5967-5971.
13. Lee JS, Song CH, Kim CH et al.: *Profiles of IFN-γ and its regulatory cytokines (IL-2, IL-18 and IL-10) in peripheral blood mononuclear cells from patients with multidrug-resistant tuberculosis*. Clin. Exp. Immunol., 2002, 128: 516-524.
14. Esin S, Batoni G, Saruhan-Direskeneli G et al.: *In Vitro Expansion of T-Cell-Receptor Vα2.3+ CD4+ T Lymphocytes in HLA-DR17(3), DQ+ Individuals upon Stimulation with Mycobacterium tuberculosis*. Infect. Immun., 1999, 67: 3800-3809.
15. Hodson WS, Luzze H, Hurst TJ et al.: *HIV-1-related pleural tuberculosis: elevated production of IFN-γ, but failure of immunity to Mycobacterium tuberculosis*. AIDS, 2001, 15: 467-475.
16. Dieli F. Friscia G, Di Sano C et al.: *Sequestration of T lymphocytes to body fluids in tuberculosis: reversal of anergy following chemotherapy*. J. infect. Dis., 1999, 180: 225-228.
17. Gambon-Deza F, Pacheco-Carracedo M, Carda-Mota T, Montes-Santiago J: *Lymphocyte populations during tuberculosis infection: Vβ repertoires*. Infect. Immun., 1995, 63: 1235-1240.
18. Ohmen JD, Barnes PF, Grisso CL et al.: *Evidence for a superantigen in human tuberculosis*. Immunity, 1994, 1: 35-43.
19. Peters W, Ernst JD: *Mechanisms of cell recruitment in the immune response to Mycobacterium tuberculosis*. Microbes and Infection, 2003, 5: 151-158.