SHORT COMMUNICATION

Flow Cytometry as a Tool to Identify *Mycobacterium tuberculosis* Interaction with the Immune System and Drug Susceptibility

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Flow cytometric analysis is a useful and widely employed tool to identify immunological alterations caused by different microorganisms, including Mycobacterium tuberculosis*. However, this tool can be used for several others analysis. We will discuss some applications for flow cytometry to the study of* M. tuberculosis, *mainly on cell surface antigens, mycobacterial secreted proteins, their interaction with the immune system using inflammatory cells recovered from peripheral blood, alveolar and pleura spaces and the influence of* M. tuberculosis *on apoptosis, and finally the rapid determination of drug susceptibility. All of these examples highlight the usefulness of flow cytometry in the study of* M. tuberculosis *infection.*

Key words: tuberculosis - macrophages - drug susceptibility - cytometry

Mycobacterium tuberculosis, the etiologic agent of tuberculosis, is responsible for more deaths each year than any other single pathogen (Murray et al. 1990). The World Health Organization has established that there are about 10 million new cases of tuberculosis each year and that tuberculosis is responsible for at least 3 million deaths annually (Bloom et al. 1992, Kaufmann & van Embden 1993). Pathogenic mycobacteria, with the possible exception of the leprosy bacillus, are facultative intracellular parasites that are capable of surviving and multiplying in phagocytes. This success is most likely inherent in the unusual properties of the mycobacterial cell envelope to interact with host cells and infect and activate them. The efficacy of the live attenuated strain of *M. bovis* BCG, which is presently the only available tuberculosis vaccine, varies considerably from one population to another. This may explain the effort invested by many groups in identifying new antigens that may be used to stimulate an effective immune defense response against *M. tuberculosis* infection. Further characterization of cell envelope antigens and the mycobacteria-macrophage interaction with the whole bacilli would help to improve our understanding of the physiopathology of tuberculosis. In this paper we will show the utility and basic uses of a modern tool – flow cytometric analysis and identify the possible role of these methods in *M. tuberculosis* infection.

Cytometric detection of mycobacterial surface antigens - The physical arrangement of cell envelope components leads to the exposure of selected structural motifs which in turn influence host-parasite interactions. To gain insight into the exposed epitopes, several authors have studied the secreted proteins for *M. tuberculosis* using flow cytometric methods designed to define molecules on dispersed mycobacteria. One of the most interesting methods is labeling with a hydrophobic fluorophore *N*-hexadecanoyl aminofluorescein (HEDAF, γ emission 520 nm) a probe that could be inserted into the mycobacterial cell envelope, which is rich in lipids. The labeled single cell population subsequently can be coated with specific monoclonal antibodies (MAbs). These MAbs can be reveled using biotinylated anti-mouse antibodies, with in turn are reacted with another fluorescent probe, fluorochrome coupled streptavidin (tricolor, γ emission 667 nm). Bacteria were first gated in a well defined morphological region in which cells stained by HEDAF were selected with FL1. The HEDAF-labeled bacteria were then analyzed for the presence of the MAb by tri-color with FL3. Thus, this method was applied to identify the relative amount of surface-exposed

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mannosylated compounds and *D*-arabinan-containing substances of different strains of tubercle bacillus (Ozanne et al. 1996). Using this strategy other cell wall constituents can be identified on the surface of mycobacteria. Differences in the location of individual proteins in relation to the mycobacterial cell are expected to be closely correlated to their functional properties and to exert an influence upon the interaction between multiplying bacilli and the infected host cells. To achive this aim a MAb against MPB83 protein, a highly homologous protein in the *M. tuberculosis* complex, was used to label *M. bovis* bacteria and could easily be reveled with fluorescein isothiocyanate-labeled MAb to mouse immunoglobulin G when run in a flow cytometry assay (Harboe et al. 1998). All sera and antibody solutions, should be sterile to avoid any particulate other than bacilli.

Interaction of M. tuberculosis with macrophages - During primary infection of the host, the first professional phagocytes encountered by *M. tuberculosis* are the alveolar macrophages (AM). *M. tuberculosis* enters and survives in the AM, subsequently, the bacilli disseminate from the lung and are phagocyted by a heterogeneous group of tissue macrophages. Indeed, phenotypic analysis of alveolar macrophages showed immature macrophages in the lung from active tuberculosis patients (Lapa-Silva et al. 1996). Little is known of the early interactions of microbes and the immune cells that results in either restricted infection or dissemination and disease. Although induction of *in vitro* murine macrophage anti-*M. tuberculosis* activity has been observed, human macrophages can achieve anti-*M. tuberculosis* activity only in a particular way, by inducing IFN-γ production by *M. tuberculosis*-specific CD4 T lymphocyte (Bonecini-Almeida et al. 1998a). The interaction between the macrophage and *M. tuberculosis* is mediated by a variety of macrophage membrane-associated proteins, such as, mannose receptors. It can be expected that cell-wall mannosylated glycoconjugates (ManLAM) mediate the binding of mycobacteria to macrophages. In an attempt to study this binding by flow cytometry, Venisse et al. (1995) reported that ManLAM could be covalently coupled to biotinamidohexanoyl, followed by incubation with streptavidin coupled to a fluorochrome which allowed its detection at the macrophage cell surface. Moreover other receptors are also being investigated. The complement receptors on macrophage are responsible for mycobacteria adhesion. Recently, Zabaleta et al. (1998) enumerated these receptors on the surface of macrophages from tuberculosis patients using monoclonal antibodies (CR1/CD35 and CR3/CD11b/CD18), trying to correlate the expression of complement receptors and the adherence and/or ingestion of *M. tuberculosis* by human macrophages. Phagocytosis of fluorescent coated (inert) particles by phagocytic cells can be rapidly enumerated by flow cytometry. Interestingly, *M. tuberculosis* bacilli are easily coated by fluorescein, Lucifer yellow and phycoerythrin, and these fluorochromes do not interfere with bacilli viability, intracellular growth or with ability of macrophages to phagocytose coated *M. tuberculosis*, making them a efficient tool with which to study mycobacteria/macrophages interaction by cytometry (Drevets et al. 1995).

Lymphocyte and lymphocyte subset number in blood, alveolus and pleura from tuberculosis patients - The pulmonary manifestation of *M. tuberculosis* infection leads to a migration and accumulation of immune cells into the lungs and pleura space. The isolation of these inflammatory cells from the site of bacteria growth may help us to examine the cell-bacilli interaction and the cellular immune activation. The cells recovered from these fluids can be identified and used, as easily as, peripheral cells by flow cytometry. However, the size of activated lymphocytes and particularly alveolar or pleural macrophages should be analyzed with attention. The variable manifestations and the natural history of tuberculosis are determined by bacterial and host factors, including cell mediated immunity. The immune response to *M. tuberculosis* involves subpopulations of specifically sensitized $CD4^+$ and $CD8^+$ T lymphocytes. Most reports of human disease are based on findings in blood or pleural fluid. In advanced or disseminated tuberculosis circulating T cells, especially $CD4^+$ cells, are reduced in number and $CD8^+$ increased relatively (Ainsle et al. 1992). We have compared lymphocyte numbers and subsets in the lungs and blood from patients with active pulmonary tuberculosis co-infected or not with HIV-1 (Bonecini-Almeida et al. 1998b,c), marking these fluid derived and peripheral cells with monoclonal antibodies against CD3, CD4, CD8, CD19, CD16/CD56. HLA-DR, IL-2Rα, transferrin-R, CD83, CD86, CD1a and CD45RO was allow them to be used as cell activation markers and to distinguish different profiles of lymphocytes, as well as, monocytes/macrophages in peripheral blood, lung and/or pleural infection by *M. tuberculosis* (Ceyhan 1996, Esin et al. 1996, Howard et al. 1998). The expression of cytokine receptors on inflammatory cells may be easily assayed by flow cytometry (Meddows-Taylor et al. 1998).

Apoptosis - Cells die by one of two known mechanisms, necrosis or apoptosis (Vaux 1993). Lysis of the host cell is predominantly through a necrotic mechanism based on morphological criteria, and did not affect *M. bovis* BCG viability (Molloy et al. 1994). In addition, perforin, a cytotoxic molecule which

mediates cell necrosis, is not required for the early control of BCG in mice (Laochumroonvoropong et al. 1997). Apoptosis plays an essential role in the development and homeostasis of multi-cellular organisms. Some infectious agents interfere with this programmed cell death to their own benefit. There is a growing awareness that apoptosis can be induced or inhibited in certain host cell-pathogen interactions (Zychlinsky 1993). Killing of macrophages by apoptosis may allow microorganisms, such as *M. bovis* (Molloy 1994), *M. avium* (Gan 1995), *M. tuberculosis* (Keane 1997), to escape from the intracellular microenvironment and to thereby disseminate to other cells or tissues. Mechanisms which would reduce the potential for macrophages to serve as protected sanctuaries for tuberculosis might be of significant benefit to the host. Conversely, efficient killing of alveolar macrophages could favor the mycobacteria by reducing the numbers of phagocytes and possibly interfering with the induction of cell mediated immunity.

Drug susceptibility - In the past decade, antimicrobial agent-resistant infections have become rampant worldwide, increasing the morbidity, mortality, and cost associated with disease (Cohen 1992). Modern clinical applications of cytometry include the determination of the most powerful drug susceptibility tests, described recently for several microorganisms, such as rickettsia (Kelly et al. 1995), fungi (Wenisch et al. 1997), *Escherichia coli* (Walberg et al. 1997), cytomegalovirus (Lipson et al. 1997), *Chlamydia* (Dessus-Babus 1998) or even testing a computer software for malaria drug susceptibility (Reinders 1995). Antimicrobial susceptibility testing for tuberculosis takes weeks and delayed therapy may compromise the patients health, increase the disease incidence and transmission to close contacts. Efforts to control tuberculosis are directly related to the time required for bacilli growth, identification and susceptibility testing which can take between two to eight weeks, primarily because of the slow growth of pathogenic bacteria (doubling times of 15 to 24 h). Recently, a rapid (24 h) method was described to identify drug susceptibility using an avirulent laboratory strain of *M. tuberculosis*-H37Ra (Norden et al. 1995). The method is based on the ability of fluorescein diacetate (FDA) to pass through the hydrophobic cell membrane of viable mycobacteria and be rapidly hydrolyzed to free fluorescein by increasing esterase. As the fluorescein accumulates in the bacteria cells, the fluorescent mycobacteria can be easily detected by flow cytometry analysis. In contrast, dead mycobacteria or mycobacteria inhibited by antimycobacteria agents hydrolyze significantly less FDA. Gates were established for viable and dead mycobacteria on the basis of their incorporation of FDA. Two parameters were evaluated: events per minute (number of labeled mycobacteria) and the mean channel fluorescence (intensity of fluorescence-labeled mycobacteria). Other nontuberculosis mycobacteria, including *M. avium, M. fortuitum, M. gordonae* or *M. marinum* were tested using the same methods, and demonstrated that the flow cytometry assay is simple, reproducible and rapid (Bownds 1996). Although the feasibility of using flow cytometry and FDA staining for susceptibility testing of *M. tuberculosis* was demonstrated, the results were obtained with an attenuated strain of *M. tuberculosis* or nontuberculosis bacilli. Furthermore, comparing the conventional agar test and flow analysis, now using clinical isolates of *M. tuberculosis* stained with FDA, Kirk et al. (1998) demonstrated an agreement between these two methods yet, there are several concerns regarding the use of flow cytometry for susceptibility testing of *M. tuberculosis*. Biosafety is frequently considered the most important. Although the test is rapid, accurate, and reproducible, many laboratories do not have the facilities to safely perform the procedure. In the past months, however, a biologically safe flow cytometry method was described. The clinical isolates of *M. tuberculosis* were exposure to drug free or antimycobacterial agent-containing medium and FDA, and subsequently inactivated by paraformaldehyde before analysis with a flow cytometry. Agreement between the results from the two methods was 98% (Moore et al. 1999). Now, it will be necessary to validate this rapid drug susceptibility method comparing clinical isolates obtained from patients in routine laboratories worldwide.

REFERENCES

Ainslie GM, Solomon JA, Baterman ED 1992. Lymphocyte subset numbers in blood and in bronchoalveolar lavage and pleural fluid in various forms of human pulmonary tuberculosis at presentation and during recovery. *Thorax 47*: 513-518.

Bonecini-Almeida MG, Chitale S, Boutsikakis I, Geng J, Doo H, He S, Ho J 1998a. Induction of *in vitro* human macrophage anti-*Mycobacterium tuberculosis* activity: requirement for IFN-γ and primed lymphocytes. *J Immunol 160*: 4490-4499.

Bonecini-Almeida MG, Werneck-Barroso E, Carvalho CP, Andrade E, Kritski A, Morgado MG 1998b. Functional activity of alveolar and peripheral cells in patients with human acquired immunodeficiency syndrome and pulmonary tuberculosis. *Cell Immunol 190*: 112-120.

Bonecini-Almeida MG, Lapa-Silva JR, Kristski A, Neves Jr I, Morgado M, Nathan C, Ho JL 1998c. Immune response during HIV and tuberculosis co-infection. *Mem Inst Oswaldo Cruz 93*: 399-402.

Bloom BR, Murray CJ 1992. Tuberculosis: commentary on a reemergent killer. *Science 257*: 1055-1064.

Bownds SE, Kurzynski TA, Norden MA, Dufek JL, Schell RK 1996. Rapid susceptibility testing for non-tuberculosis mycobacteria using flow cytometry. *J Clin Microbiol 34*: 1386-1390.

Ceyhan BB, Demiralp E, Celikel T 1996. Analysis of pleural effusions using flow cytometry. *Respiration 63*: 17-24.

Cohen ML 1992. Epidemiology of drug resistance: implications for a post-antimicrobial era. *Science 25*: 1050-1055.

Drevets DA, Elliott AM 1995. Fluorescence labeling of bacteria for studies of intracellular pathogenesis. *J Immunol Methods 18*: 69-79.

Dessus-Babus S, Belloc F, Bebear CM, Poutiers F, Lacombe F, Bebear C, de Barbeyrac B 1998. Antibiotic susceptibility testing for *Chlamydia trachomatis* using flow cytometry. *Cytometry 31*: 37-44.

Esin S, Batoni G, Källenius G, Gaines H, Campa M, Svenson SB, Andersson R, Wigzell H 1996. Profilation of distinct human T cell subsets in response to live, killed, or soluble extracts of *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Clin Exp Immunol 104*: 419-425.

Gan H, Newman GW, Remold HG 1995. Plasminogen activator inhibitor type 2 prevents programmed cell death of human macrophages infected with *Mycobacterium avium*, serovar 4. *J Immunol 155*: 1304-1315.

Harboe M, Wiker HG, Ulvund G, Lund-Pedersen B, Andersen AB, Hewinson RG, Nagai S 1998. MPB70 and MPB83 as indicators of protein localization in mycobacterial cells. *Infect Immun 66*: 289-296.

Howard AD, Trask OJ, Weisbrode SE, Zwilling BS 1998. Phenotypic changes in T cell populations during the reactivation of tuberculosis in mice. *Clin Exp Immunol 111*: 309-315.

Kaufmann SH, Van Embden JD 1993. Tuberculosis: a neglected disease strikes back. *Trends Microbiol 1*: 2-5.

Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ, Kornfeld H 1997. Infection by *Mycobacterium turbeculosis* promotes human alveolar macrophage apoptosis. *Infect Immun 65*: 298-304.

Kelly DJ, Salata KF, Strickman D, Hershey JN 1995. Rickettsia tsutsugamushi infection in cell culture: antibiotic susceptibility determined by flow cytometry. *Am J Trop Hyg 53*: 602-606.

Kirk MK, Schell RF, Moore AV, Callister SM, Mazurek GH 1998. Flow cytometry testing of susceptibilities of *Mycobacterium tuberculosis* isolates to ethambutol, isoniazid, and rifampicin in 24 hours. *J Clin Microbiol 36*: 1568-1573.

Laochumroonvoropong P, Wang J, Liu C-C, Ye W, Moreira AL, Elkon KB, Freedman VH, Kaplan G 1997. Perforin, a citotoxic molecule which mediates cell necrosis, is not required for the early control of mycobacterial infection mice. *Infect Immun 65*: 127-132.

Lapa-Silva JR, Linhares C, Boechat N, Rego L, Bonecini-Almeida MG 1996. Phenotypes of lung mononuclear phagocytes in the bronchoalveolar lavage fluid of HIV-1 seronegative tuberculosis patients: evidence for new recruitment and cell activation. *Mem Inst Oswaldo Cruz 91*: 389-394.

Lipson SM, Soni M, Biondo FX, Shepp DH, Kaplan MH, Sun T 1997. Antiviral susceptibility testing-flow cytometric analysis (AST-FCA) for the detection of cytomegalovirus drug resistance. *Diagn Microbiol Infect Dis 28*: 123-129.

Meddows-Taylor S, Martin DJ, Tiemessen CT 1998. Reduced expression of interleukin-8 receptors A and B on polymorphonuclear neutrophils from persons with human immunodeficiency virus type 1 disease and pulmonary tuberculosis. *J Infect Dis 177*: 921-930.

Molloy A, Laochumroonvorapong P, Kaplan G 1994. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guérin. *J Exp Med 180*: 1499-1509.

Moore AV, Kirk SM, Callister SM, Mazurek GH, Schell RF 1999. Safe determination of susceptibility of *Mycobacterium tuberculosis* to antimycobacterial agents by flow cytometry. *J Clin Microbiol 37*: 479-483.

Murray CJ, Styblo K, Rouillon A 1990. Tuberculosis in developing countries: burden, intervention and cost. *Bull Int Union Tuberc Lung Dis 65*: 6-24.

Norden MA, Kurzynski TA, Bownds SE, Callister SM, Schell RF 1995. Rapid susceptibility testing of *Mycobacterium tuberculosis* (H37Ra) by flow cytometry. *J Clin Microbiol 33*: 1231-1237.

Ozanne V, Ortalo-Magne A, Vercellone A, Fournie JJ, Daffe M 1996. Cytometric detection of mycobacterial surface antigens: exposure of mannosyl epitopes and of the arabinan segment of arabinomannans. *J Bacteriol 178:* 7254-7259.

Reinders PP, van Vianen PH, van der Keur M, van Engen A, Janse CJ, Tanke HJ 1995. Computer software for testing drug susceptibility of malaria parasites. *Cytometry 19*: 273-281.

Vaux DL 1993. Toward an understanding of the molecular mechanisms of physiological cell death. *Proc Natl Acad Sci USA 90*: 786-789.

Venisse A, Fournie JJ, Puzo G 1995. Mannosylated lipoarabinomannan interacts with phagocytes. *Eur J Biochem 231*: 440-447.

Walberg M, Gaustad P, Steen HB 1997. Rapid assessment of ceftazidime, ciprofloxacin, and gentamicin susceptibility in exponentially-growing *E. coli* cells by means of flow cytometry. *Cytometry 27*: 169-178.

Wenisch C, Linnau KF, Parschalk B, Zedtwitz-Liebenstein K, Georgopoulos A 1997. Rapid susceptibility testing of fungi by flow cytometry using vital staining. *J Clin Microbiol 35*: 5-10.

Zabaleta J, Arias M, Maya JR, Garcia LF 1998. Diminished adherence and/or ingestion of virulent Mycobacterium tuberculosis by monocyte-derived macrophages from patients with tuberculosis. *Clin Diagn Lab Immunol 5*: 690-694.

Zychlinsky A 1993. Programmed cell death in infectious diseases. *Trends Microbiol 1*: 114-117.