Immunological Evaluation of Human Immunedeficiency Virus Infected Individuals by Flow Cytometry

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Human immunodeficiency virus (HIV) infection heavily compromises the immune system. The decrease of the T cell CD4+ subset along the evolution to acquired immunodeficiency syndrome has been considered as a hallmark of HIV infection. In this paper we review some aspects of the immunopathology of HIV infection and discuss the importance of the flow cytometry for the evaluation of the T lymphocyte subsets in the follow-up of HIV infected children and adults, and for the monitoring of the immune reconstitution upon antiretroviral therapy.

HIV INFECTION

The human immunodeficiency virus (HIV) infection heavily compromises the immune system leading to opportunistic infections, neoplasias and neurological commitments. The drop of the T cell CD4^{+} subset along the evolution to acquired immunodeficiency syndrome (Aids) is a hallmark of HIV infection. Indeed, this major alteration of the immune system was quickly associated with the clinical observations permitting the staging of the patients along the evolution to Aids. The CD4^{+} T lymphocyte counts has been considered as a surrogate marker for disease progression, as well as for the antiretroviral therapy and prophylaxis for opportunistic pathogens (Stites et al. 1989, Bogner & Goebel 1991, Fauci 1993). Moreover, the CD4^{+} T lymphocyte counts may also be used as surrogate markers of HIV infection in patients who have delays in HIV serologic reporting or have refused to test. There are relatively few conditions associated with the profound depletion of this T cell subset in patients who have the classical "Aids-defining diagnoses", included in their differential diagnosis (reviewed by Bartlett 1998). More recently, the viral load measurement was introduced to the routine laboratorial evaluation of HIV infected individuals and this parameter was shown to be of paramount importance to predict time to Aids or death, which increase with a steeper CD4 decline and a higher viral burden (Mellors et al. 1996,

1997, Fahey et al. 1998).

At least four profiles can be distinguished among HIV infected people concerning the progression to disease (Fauci 1993, Pantaleo & Fauci 1996). The majority of them (70-80%) progress to Aids in five to ten years (typical progressors), with progressive loss of CD4^{+} T lymphocyte and immune functions and persistent virus replication. In a small percentage, some individuals progress within a period similar to the typical progressors, but the clinical, immunological and virological parameters remain stable for a longer period of time (long-term survivors). Another significant percentage of individuals (10-15%), however, rapidly progresses to Aids and death within the first 2-3 years after seroconvertion (rapid progressors). On the other hand, a small percentage (less than 5%) do not experience the typical loss of CD4^{+} T lymphocytes over an extended period of time and are described as long-term nonprogressors. The HIV positive individuals included in this group have low levels of virus, and preservation of lymphoid tissue architecture and immune functions. Although the mechanisms favoring one or another type of progression are not completely clarified, host genetic profile, immune responses, as measured, for example, by the T cell repertoire usage (Pantaleo et al. 1994, 1997, Graziosi et al. 1998) or the cytokines and chemokines production and regulation (Cohen et al. 1997), as well as the virus biological and genetical profiles are certainly contributing to the distinct patterns of progression (reviewed by Fauci 1996).

The recent advent of potent antiretroviral combination therapy, inducing profound and rapid falls in the levels of plasma virus, has been changing the course of Aids, and giving a new insight for

controlling the progression to disease. However, discontinuation of antiretroviral therapy after short or prolonged (Stellbrick et al. 1996, Finzi et al. 1997, Connors et al. 1997) treatment of chronically infected patients, keeping plasma viral load below detectable levels, results in rapid increase of viremia, indicating persistent active virus replication even in the presence of the drugs.

FLOW CYTOMETRY AND HIV INFECTION

Although the laboratorial hallmark of the evolution of HIV infection is the T cell CD4$^+$ subset reduction over time, functional studies have demonstrated qualitative impairment of the CD4$^+$ lymphocyte functions occurring early in the course of infection, even before its quantitative decrease (Rosenberg & Walker 1998). Indeed, CD4$^+$ helper T cell responses are known to be essential for maintenance of the effective immunity in chronic viral infections and the hierarchical loss of the CD4$^+$ functional activity, measured both by the reduction in the lymphoproliferative response and the IL-2 secretion, has been demonstrated in HIV-1 infected patients even before Aids onset (Clerici et al. 1989). In a first stage, loss of T cell response to common recall antigens (FLU, TT), followed by alloantigens and mitogens can be prospectively observed, however, these methodologies are very time consuming and need special laboratory facilities for the monitoring of HIV infected patients.

The development of a system permitting the automatic count of particles suspended in a fluid was patented in 1949 (Coulter 1949), and it took almost twenty years to evolve to a computer assisted multiparametric flow cytometric data analysis system (Dittrich & Gohde 1968), which has achieved paramount importance within the range of clinical pathology procedures and research applications.

Flow cytometry is the standard method for determining CD4$^+$ T lymphocytes counts in order to monitor HIV infection. Associated with the usage of specific monoclonal antibodies labeled with several available fluorocromes, this methodology permits a specific and fast semi-automatic quantification of cellular subsets, and the processing and reading of several samples in a short time. Other alternative methodologies have also been developed over time, like (a) the FACS Count System (Becton Dickinson Immunocytometry Systems), (b) VCS Technology/Coulter Cyto-Spheres (Coulter Corporation), (c) Zymmune CD4/CD8 Cell Monitoring Kit (Zynaxis, Inc) and (d) TRAx CD4 Test Kit (T Cell Diagnostics), which present a good performance when compared with standard flow cytometry and require less technical expertise (Johnson et al. 1995). However, different from standard flow cytometry, these alternative systems are only restricted to the CD4$^+$ and CD8$^+$ T lymphocyte subsets quantification.

The advance of the flow cytometry for two, three or four-color systems allowed the analysis of distinct surface molecules in a single cell, leading to more accurate evaluation of specific cell subsets. A typical panel composed of fluorescein isothiocianate (FITC) and phycoerytrin (PE/RD1) labeled monoclonal antibodies is among the most frequently used for routine analysis for the monitoring of the HIV infection. Current standards use multiple tube assay including CD45$^+$/CD14$^+$ as gating control, isotype control, CD3$^+$/CD4$^+$ and CD3$^+$/CD8$^+$ double labeled monoclonal antibodies (CDC 1994).

The technical improvement for the three-color system, using the combination of PerCP (peridinin chlorophyll protein), FITC and PE labeled monoclonal antibodies allows the determination, for example, of the CD3$^+$/CD4$^+$, CD3$^+$/CD8$^+$ subsets and the total CD3$^+$ in a single tube (Fig. 1). In this case, the lymphocyte region is determined based on the side scatter (SS) and forward scatter (FS) histogram. Moreover, with the four-color staining system, the leucogate histogram can be based on the SS and the FITC labeled CD45$^+$ and the T cell subsets are immunophenotyped using CD3$^+$-PC5, CD8$^+$-ECD (phycoerythrin-Texas Red) and CD4$^+$-RD1 labeled monoclonal antibodies in a single tube assay (Fig. 2). In this case, the lymphocytes are phenotyped as low granulated CD45$^{+bright}$ expressing cells. In addition to the higher accuracy of these three or four-color systems, one other advantage is the low quantity of blood employed (100 µl/assay), which is very relevant for Aids pediatrics, as well as for multiple immunological and virological analysis of HIV positive individuals combining routine exams and research purposes.

Due to the several aspects that can interfere in the flow cytometric analysis of the T cell subsets of HIV infected individuals, it is of paramount importance to establish quality control procedures in order to guarantee the accuracy of the final results. Another important issue with regard to cytometry assay with specimens from HIV-infected individuals is biohazard considerations. For the protection of the cytometryst, specimens can be fixed with a 1% solution of formaldehyde or paraformaldehyde, and the waste container system should be used with a final concentration of 1% sodium hypochlorite in order to inactivate the HIV.

CD4$^+$ T CELL INTERPRETATION

Age dependent differences have been described for the CD4$^+$ T cell count analysis, and this variation has to be taken into consideration for prophy-

lactic and antiretroviral therapies of HIV infected individuals.

For adults and adolescents the normal values of CD4$^+$ T lymphocytes in the bloodstream vary from 800-1050/mm^3, with a range representing two standard deviations of approximately 500 to 1400/mm^3 (Laurence 1993). Such variability reflects the variables used for the CD4$^+$ T lymphocyte determination which takes into account the leukocyte counts, the percentage of lymphocytes and the percentage of lymphocytes that bear the CD3$^+$ and CD4$^+$ receptors. Seasonal, month-to-
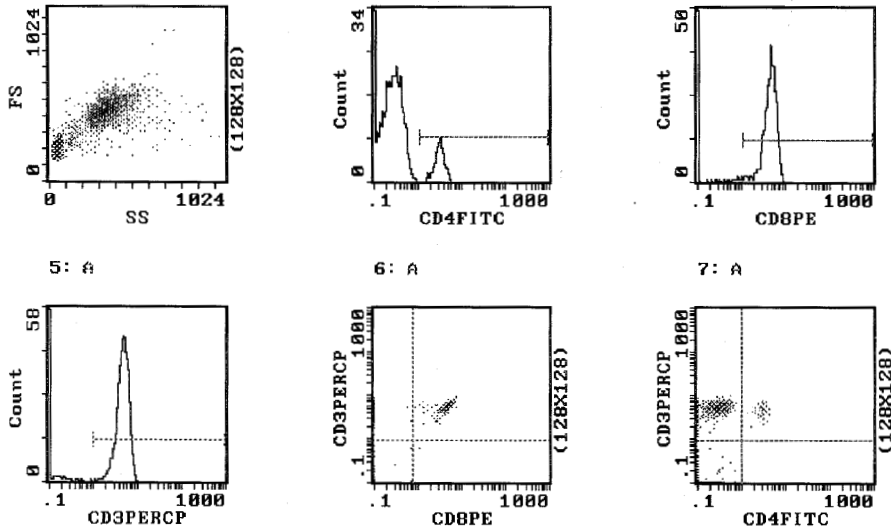

Fig.1: three color cell surface marker analysis. The triple color analysis indicates the CD3, CD3/CD4 and CD3/CD8 lymphocyte subsets from an human immunodeficiency virus (HIV)-positive individual. One hundred microlitres of whole blood was incubated for 30 min at room temperature in the dark with 10 μl of monoclonal antibodies (Tritest CD4FITC/CD8PE/CD3PerCP, Becton Dickinson CO, USA) and automatically lysed and fixed using a Multi-Q-Prep system, according to the manufacturer (Coulter Corp., Healeah, Fl, USA). The T cell subset determinations were carried out using an EPICS XL-MCL Flow Cytometer (Coulter Corp., Healeah, Fl, USA).
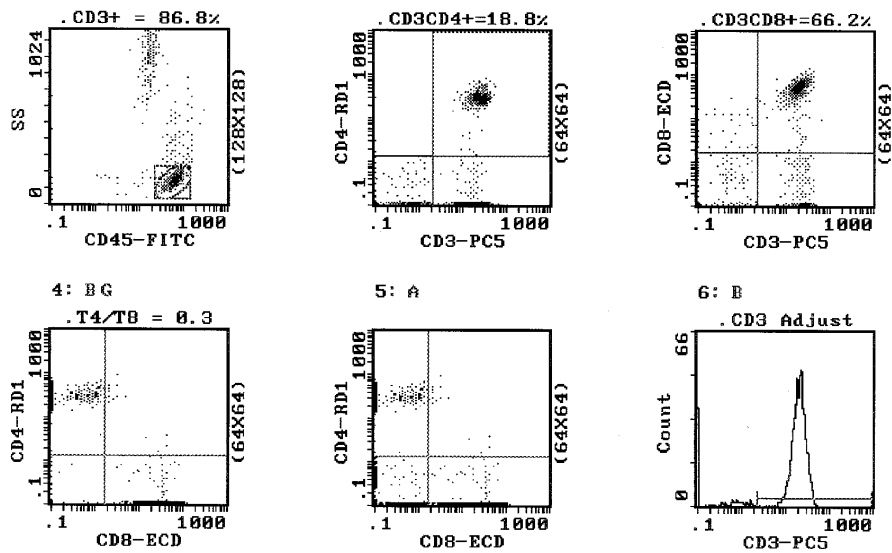

Fig. 2: four color cell surface marker analysis. The four color analysis indicates the CD45, CD3, CD3/CD4 and CD3/CD8 lymphocyte subsets from an human immunodeficiency virus (HIV)-positive individual. One hundred microlitres of whole blood was incubated for 30 min at room temperature in the dark with 10 μl of monoclonal antibodies (Cyto-Stat tetraCHROME, CD45FITC/CD4RD1/CD8ECD/CD3PC5, Coulter Co, Miami, FL, USA) and automatically lysed and fixed using a Multi-Q-Prep system, according to the manufacturer (Coulter Corp., Healeah, Fl, USA). The filters used for the four-color analysis were 525 BP, 575 BP, 620 BP, 675 BP and the T cell subset determinations were carried out using an EPICS XL-MCL Flow Cytometer (Coulter Corp., Healeah, FL, USA).

month and diurnal changes in the CD4$^+$ T lymphocyte counts have been described in healthy adults (van Rood et al. 1991), whereas factors like gender, age in adults, risk category, psychological or physical stress and pregnancy have minimal effects on the CD4$^+$ T lymphocyte counts (reviewed by Bartlett 1998).

The revised CDC (Centers for Disease Control and Prevention) classification system for HIV-infected adolescents and adults (CDC 1993) is based on the clinical conditions and the CD4$^+$ T lymphocyte counts. This system is represented by a matrix of nine categories (A1 to C3) based on the combination of three levels of CD4$^+$ T lymphocyte counts (1. > 500/mm^3; 2. 200-499/mm^3; 3. <200/mm^3) and three clinical categories (A, B and C). Asymptomatic HIV infection, persistent generalized lymphadenopathy and acute HIV infection are included in category A, whereas distinct associated diseases are included in the categories B and C.

The Advisory Group on Antiretroviral Therapy of the Brazilian STD/Aids Program (Ministério da Saúde 1998) has also established a system to categorize HIV infected individuals in order to recommend initiation of antiretroviral and prophylactic therapy in the country, as well as the therapeutic regime to be applied depending on the clinical, immunological and virological conditions. Brazil is one of the few countries in the world offering antiretroviral and prophylactic treatment, as well

as monitoring exams as CD4$^+$ T lymphocyte counts and viral load measurement for HIV infected individuals. Table I shows the clinical, immunological and virological criteria considered for the recommendation of initiating antiretroviral and prophylactic treatment of HIV infected adults and adolescents in the country.

The CD4$^+$ T lymphocyte counts in young healthy children (less than 5 years old) are higher than adults, varying from around 3,000/mm^3 at 6 months old to 2,500/mm^3 and 1,500/mm^3, respectively, at 12 and 24 months old (Erkeller-Yuksel et al. 1992). Similar values to adults can be achieved in children who are 6 years old or more, and this normal decay in the CD4+ T lymphocyte population in younger children has to be taken into consideration for the analysis of the immune alterations in Aids pediatrics. Indeed, the CDC classification for Aids pediatrics is based both in the clinical signs and symptoms (N: without signs and symptoms; A, B and C, respectively, light, moderate and severe signs and symptoms) and the immunological alterations, defined as absent (1), moderate (2) and severe (3), based on the CD4$^+$ T lymphocyte counts (CDC 1994). Table II presents these levels of immunological alterations considered for HIV-infected children (0-12 years old). Similarly to what has been described for adults and adolescents, this system classification is represented by a matrix of twelve categories (N1 to C3) based on clinical and immunological parameters. The STD/Aids Pro-

TABLE I

Recommendations of the Brazilian STD/Aids Program for initiating therapy of human immunodeficiency virus (HIV) infected adults and adolescents

Clinical situation	CD4 (cells/mm^3) cytometry	Count viral load (copies/ ml) flow	Recommendation
Asymptomatic	Not available	Not available	No treatment
Asymptomatic	≥ 500	1. <100.000 2. ≥100.000 3. Viral load not available	1. No treatment 2. Consider treatment 3. No treatment
Asymptomatic	≥ 350 < 500	1. < 30.000 2. ≥ 30.000 2. Treat	1. Consider treatment
Asymptomatic	≥200 < 350	1. < 30.000 2. ≥ 30.000	1.Treat 2. Treat
Asymptomatic	< 200	Irrespective of viral load	Initiate therapy and prophylaxis for opportunistic infections

Whenever there are no CD4 cell counts or viral load evaluations available, use of antiretrovirals is recommended for symptomatic patients only, except for special situations: pregnant HIV-infected women, newborn of HIV-positive mother and occupational exposure to HIV. Adapted from the guidelines for clinical treatment of HIV infection in adults and adolescents, 1999, National Coordination of STD and Aids, Brazilian Ministry of Health, in portuguese. (http://www.Aids.gov.br) for updating.

TABLE II

Immunological categories   of human immunodeficiency virus (HIV)-infected children based on age and $CD4^+$ T lymphocyte counts/mm^3 of peripheral blood

Immunological alterations	Age $CD4^+$ lymphocyte counts/mm^3 (flow cytometry)		
	<12 months	1 to 5 years old	6 to 12 years old
Absent (1)	> 1,500 (> 25%)	>1,000 (> 25%)	> 500 (> 25%)
Moderate (2)	750-1,499(15- 24%)	500-900 (15-24%)	200-499 (15-24%)
Severe (3)	< 750 (< 15%)	500 (< 15%)	< 200 (< 15%)

CDC (1994), reproduced from the guideline for clinical treatment of HIV infection in children, 1998, National Coordination of STD and Aids, Brazilian Ministry of Health, in portuguese; http://www.aids.gov.br for updating.

gram from the Brazilian Ministry of Health use this system to base the consensus recommendation for antiretroviral therapy in HIV infected children (guideline for clinical treatment of HIV infection in children, 1998, National Coordination of STD and Aids, Brazilian Ministry of Health, in Portuguese).

$CD8^+$ LYMPHOCYTES AND HIV INFECTION

The importance of the $CD4^+$ T lymphocyte counts has been rapidly incorporated to the clinical and laboratorial monitoring of HIV infected people, however the evaluation of other subsets of T lymphocytes, characterized by a wide panel of monoclonal antibodies already available, which could delineate in a better extent the immunological status of HIV infected individuals, is still restricted to research protocols.

Changes in the $CD8^+$ T lymphocytes subsets, associated with progressive inversion in the $CD4^+$/$CD8^+$ ratio have been studied as markers of disease progression in HIV infected people (Ferbas 1998). Increase in the absolute number of $CD8^+$ T lymphocyte over time is a well known event and is not correlated with reduction on viral load. Moreover, increased expression of $CD38^+$ and HLA-DR^+ cell activating markers in $CD8^+$ T lymphocytes from HIV infected people progressing to Aids has also been described (Kestens et al. 1992). Fig. 3 is representative of the evaluation of $CD8^+$/$CD38^+$ T cell activated lymphocytes, using a two-color panel with $CD8^+$-FITC and $CD38^+$-PE labeled monoclonal antibodies (Neves Jr et al. unpublished observations). The HIV-1 seropositive individuals included in this study are being analyzed before and after the introduction of the highly active antiretroviral therapy (HAART), in order to evaluate its effect on T cell activation and apoptosis (data not shown), among other clinical, immunological and virological parameters. Reduction on the $CD8^+$ T cell subset during antiretroviral therapy has been previously documented (Autran et al. 1997), including a decrease on the CD38 expres-

sion on $CD8^+$ lymphocytes (Bouscarat et al. 1998). However , undetectable plasma HIV RNA is not associated with a return to normal $CD8^+$ lymphocyte activation status even after six months of treatment, suggesting that viral replication persist in lymphoid tissues.

The presence of virus-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) can be detected during early HIV infection and has been correlated with downregulation of viremia after the acute viral syndrome (Walker & Levy 1989). Moreover, in addition to regulatory and pro-inflamatory cytokines, $CD8^+$ T lymphocytes release β-chemokynes, as RANTES, MIP-1α and MIP1-β, which are the natural ligands of the CCR5 co-receptor for virus cell entry (Cocchi et al. 1995, Deng et al. 1996, Dragic et al. 1996, Alkhatib et al. 1996), as well as the cell activating factor, CAF (Levy et al. 1996), described as a soluble factor

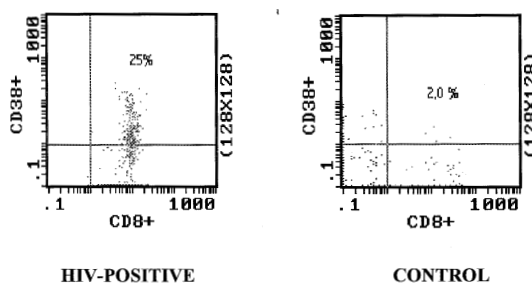**HIV-POSITIVE**   **CONTROL**

Fig. 3: phenotyping changes of CD8 lymphocytes during human immunodeficiency virus (HIV)-1 infection. Three-color flow cytometric analysis shows high expression of the activation marker CD38 on CD3/CD8 T lymphocytes from an HIV positive individual compared to a normal control. One hundred microlitres of whole blood was incubated for 30 min at room temperature in the dark with 10 ml of the monoclonal antibodies CD3FITC/CD8RD1 (Coulter Corp., Healeah, FL, USA) and CD38Cy-Chrome (Pharmigen, CA, USA) and automatically lysed and fixed using a Multi-Q-Prep system, according to the manufacturer (Coulter Corp., Healeah, FL, USA). The T cell subset determinations were carried out using an EPICS XL-MCL Flow Cytometer (Coulter Corp., Healeah, FL, USA).

inhibitor for *in vitro* virus replication in HIV-infected $CD4^+$ T cells. Therefore, $CD8^+$ T lymphocytes are important anti-viral components in the immunopatology of HIV infection.

ANALYSIS OF CELLULAR DEATH IN HIV INFECTION

The suicide programmed cell death, called apoptosis occurs under different physiological and pathological conditions. In HIV infection apoptosis has been widely described along the progression to Aids in freshly obtained peripheral blood lymphocytes or purified cells upon antigenic or mitogenic stimulation, affecting both $CD4^+$ and $CD8^+$ T lymphocyte subsets (reviewed by Ameisen 1994). Indeed, apoptosis in HIV infected individuals in an early marker occurring independently of high viremia (Rothen et al. 1997). More recently, significant decrease in apoptosis of lymphoid tissue (Badley et al. 1998), and in the memory T cell subset (Gougeon et al. 1999), has been observed in HIV infected individuals submitted to HAART. The analysis of cellular death can be determined by several approaches. Flow cytometric analysis of the propidium iodide stained lymphocytes can easily be performed to determine cell death and cellular cell cycle (Fig. 4), where the degree of fluorescence represents the amount of the DNA, which varies along the cell cycle (Braylan et al. 1982, Hamel et al. 1996, Neves Jr et al. 1998). Although the simultaneous PI and cell surface labeling has been described (Garvy et al. 1993), this method could be used with precaution, regarding the lost of cell membrane integrity. The 7-aminoactinomicin (7-aad), however, is an alternative DNA dye which allows the discrimination of apoptosis and necrosis in cell populations (Rabinovitch et al. 1986). Moreover, it is possible to combine a three-color system, including 7-aad staining associated with specific PE and FITC labeled cell surface monoclonal antibodies, permitting the discrimination among the targeted cells (Schmid et al. 1992). In addition, the HO342 (Bisbenzimidazole), a vital dye for identification of live and apoptotic cells, is a third alternative dye, giving similar results of 7-aad in the apoptosis determination (Schmid et al. 1994). However, these techniques should not be taken as definite evidence for the presence of apoptotic cell population without other supporting information. Apoptosis, possibly mediated through the CD95 antigen, has been proposed as a mechanism for cell loss, which eventually leads to immune dysfunction. Increased $CD95^+$ expressing $CD4^+$ and $CD8^+$ T cell subsets have been observed in HIV-infected individuals (Aries et al. 1995, McCloskey et al. 1995, 1998, Baumler et al. 1996). Taken together, these data suggest that flow cytometric analysis of apoptosis
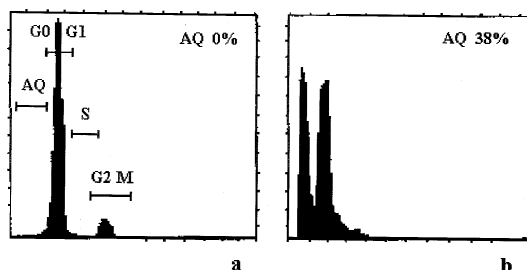

Fig. 4: flow cytometric analysis of cell cycle and programed cell death (apoptosis). Cell cycle was determined for propidum iodide (PI) stained PBMC, incubated with (a) culture medium and (b) phytohemaglutinin, obtained from an human immunodeficiency virus (HIV)-positive patient. Determination of cell cycle distribution was carried out using an EPICS 751 Flow Cytometer (Coulter Corp., Healeah, FL, USA). Apoptotic cells were determined based on the appearance of a peak in the sub-G0G1 region (b). From Neves Jr et al. (1998).

could also be included for the monitoring of the immune system in HIV infected individuals.

FINAL REMARKS

The recent introduction of combined highly active antiretroviral therapy has substantially reduced the viral load in most HIV infected people. Indeed, increase in the $CD4^+$ T lymphocyte levels, associated with the reduction of plasma viral load, have been thoroughly described in people submitted to HAART (Autran et al. 1997, 1999, Lefeuillade et al. 1997, Pantaleo 1997), presenting new questions about the potential of the reconstitution of the immune system of HIV infected individuals with distinct levels of immunosupression. In this field, flow cytometry is the methodology of choice, permitting the evaluation of several cell populations, as well as their proliferating capacity and cytokine secretion patterns (Landay et al. 1990, Krouwels et al. 1997), among other applications.

Moreover, several other applications of flow cytometry can also be depicted in the HIV/Aids field. Evaluation of seroreactivity to HIV antigens adsorbed to beads (immunoreactive bead assay), as well as the qualitative and quantitative analysis of HIV infected cells by means of the detection of p24 antigen in peripheral blood mononuclear cells, using specific monoclonal antibodies, are examples of these possibilities. A recent description of flow cytometry to evaluate the presence and specificity of cytotoxic T cells based on their reactivity to human leukocyte antigen-peptide tetrameric complexes (Ogg et al. 1998) open a new and powerful field for the monitoring of HIV infected patients, as well as for the evaluation of the cell mediated immunity induced by the HIV/Aids vaccines under analysis.

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