OPTI-CIM: A DIAGNOSTIC KIT FOR DETERMINING LYMPHOID SUBPOPULATIONS AND ITS APPLICATION IN MONITORING PATIENTS WITH HIV INFECTION

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ABSTRACT

The purpose of this study was to evaluate the Opti-CIM diagnostic kit, based on an alternative method to flow cytometry that employs light microscopy using monoclonal antibodies and alkaline phosphatase conjugated antiserums for the determination of the CD3, CD4 and CD8 lymphocyte subsets in individuals with HIV/AIDS. The Opti-CIM method compared favorably with the standard flow cytometric technology for CD4+ T-cell percentage, resulting in an overall correlation coefficient of 0.95 (95 % confidence interval, $P < 0.01$) for the 60 patients evaluated. This simple to perform, cost-effective diagnostic kit that requires no special equipment, is an attractive alternative for the determination of lymphoid subpopulations in third world countries where HIV prevalence is high and funds for flow cytometry are limited.

Key words: light microscopy, monoclonal antibodies, CD4+ T-cell

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RESUMEN

El objetivo de este trabajo es evaluar el estuche diagnóstico Opti-CIM, basado en un método alternativo a la citometría de flujo que emplea microscopía óptica usando anticuerpos monoclonales y antisueros conjugados con fosfatasa alcalina para la determinación de las subpoblaciones linfocitarias CD3, CD4 y CD8 en individuos con HIV/SIDA. El estudio comparativo del Opti-CIM con el método estándar de citometría de flujo para el porcentaje de células T CD4+ resultó en un coeficiente de correlación de 0,95 (intervalo de confianza de 95 %, $P < 0,01$) para el total de 60 muestras evaluadas. Este estuche diagnóstico simple y económico, que no requiere de equipamiento especial, constituye una alternativa atractiva para la determinación de las subpoblaciones linfocitarias en los países del tercer mundo donde la prevalencia del VIH es elevada y los recursos financieros para la citometría de flujo son escasos.

Palabras claves: microscopía óptica, anticuerpos monoclonales, CD4+ T-cell

Introduction

The evaluation of the lymphoid subpopulations has become a powerful tool for determining the immunological status in patients with autoimmune disease and other immune system related disorders, and for the diagnosis, classification and monitoring of treatment of leukemias and lymphomas (1, 2) as well as, for monitoring organ transplanted patients (3) and assessing the stage of HIV infected individuals and predicting progression to AIDS (4-9).

Among the persons with HIV infection the CD4+ T-cell level (per cent or absolute count) (5, 9) is one of the best indicators of disease progression, risk of opportunistic infections and response to therapeutic intervention (4-9).

In most laboratories the standard method for CD4+ T-lymphocyte determination involves the use of flow cytometry. This method requires expensive instruments and maintenance, as well as trained technologists, thus limiting the availability of CD4+ T-cell enumeration in third world countries (4, 6, 7).

Recently, a simple to perform, cost-effective diagnostic kit has been developed (Opti-CIM, CIMAB S.A., Havana, Cuba). This kit is based on a modification of a previously described (10, 11) light microscopy technique that utilizes monoclonal antibodies and alkaline phosphatase conjugated antiserums for the determination of CD3, CD4 and CD8 lymphocyte subsets.

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1. Faxas ME, Valdés H, Barral AM, Expóxito G, Hurtado P, Barroso MC *et al.* Ensayo fase I-II de tratamiento de los linfomas T cutáneos (LCTC) con el anticuerpo monoclonal murino IOR T1 (anti CD6): monitoreo de las pruebas de laboratorio durante el tratamiento. Biotecnología Aplicada 1993;10:20-2.

2. Tormo BR, García CA, Chong A, Ochoa C, Faxas ME, Sagaró B *et al.* Immunohistopathology of cutaneous T-cell lymphomas treated with topic IOR T1 (anti CD6) monoclonal antibody. Biotecnología Aplicada 1994;11:20-24.

Evaluation of blood samples from healthy volunteer donors demonstrated an overall correlation of the Opti-CIM assay with flow cytometry of 0.96 (unpublished data). The clinical utility of this assay is very attractive in third world countries where HIV prevalence is high and funds for flow cytometry are limited.

Methods

Design

A cross -sectional study of HIV seronegative and HIV seropositive individuals was carried out for CD3+, CD4+, CD8+ T-cell percentages by both standardized flow cytometry measurements and Opti-CIM technology using light microscopy.

Subjects

EDTA anticoagulated whole blood was obtained from 54 HIV seropositive and 6 HIV seronegative individuals for the evaluation of CD3+, CD4+, CD8+ T-cells by flow cytometry and Opti-CIM assay.

Flow cytometry

CD3+, CD4+, CD8+ T-cell percentages were determined using a whole blood lysis assay. EDTA anticoagulated blood (100 µL) was added to tubes followed by the addition of the monoclonal antibody reagent. Samples were incubated for 20 min at room temperature. Subsequently samples were incubated with FITC conjugated rabbit anti mouse reagent (DAKO A/S) at room temperature for 20 min. Finally red blood cells were lysed and fixed with 0.5-1 % formaldehyde and analyzed on an ORTHO Cytoron absolute instrument.

Antibody panel used for flow analysis included isotype controls (IgG2a, IgG2b), and anti CD3, CD4 and CD8 MAbs (CIMAB S.A., Cuba, Cat. No. CN002, CN003, SC003, SC004, SC008).

Opti-CIM assay

The Opti-CIM diagnostic kit evaluated in this study is based on a modification of a previously reported method (10, 11) developed at the Quality Control Department of the Center of Molecular Immunology (manuscript in preparation) and briefly described below:

Peripheral blood was collected in a heparinized RPMI medium (approximately equal volumes of blood and medium). Mononuclear cells were isolated by centrifugation on a Ficoll-Isopaque or a similar separation medium. Cells were counted and the cell concentration adjusted to 10^6 cells per mL PBS. Ten microliters of the cell suspension was applied to each well (circle) on the gelatin coated slides. Slides were air dried and cells incubated with 10 µL of monoclonal antibody reagent for 20 min at room temperature, followed by the addition of 10 µL of alkaline phosphatase conjugated sheep anti mouse reagent for 20 min at room temperature. Subsequently 10 µL of alkaline phosphatase conjugated rabbit anti sheep reagent was applied for 20 min at room temperature. Next 10 µL of the substrate-chromogen solution (Sigma, UK) was applied to each sample and incubated for 5 to 10 min at room temperature. Cells were fixed with 37 % formaldehyde vapors for 10 min. Nuclei were stained with methyl green solution at 37 °C for 10 min. Slides were mounted with gelatin-glycerin and positive cells counted from a minimum of 200 cells under a light microscope using 40 X or immersion 100 X lens.

Absolute CD4+ T-cell counts can be estimated as the product of the CD4+ T-lymphocyte percentage and the absolute lymphocyte count determined by a hematology analyzer.

The antibody panel used in Opti-CIM Kit (CIMAB S.A. Cat. No. J001) included anti CD3, CD4 and CD8 MAbs (CIMAB S.A., Cuba, Cat. No. SC003, SC004, SC008).

Statistical analysis

Statistical analyses were run using the software package statgraphic on a PC compatible microcomputer. Descriptive statistics (means, standard deviations and coefficients of variation), correlation coefficients r_{xy} and scattergrams were determined for the CD4+ T-cell percentages by patient serostatus. For CD4+ T-cell evaluation, sensitivity was defined as the number of individuals with CD4+ T-cell percentages < 14 % by both methods divided by the number of individuals with CD4+ T-cell percentages < 14 % by flow cytometry. Specificity was defined as the number of individuals with CD4+ T-cell percentages > 14 % by both methods divided by the number of individuals with CD4+ T-cell percentages > 14 % by flow cytometry.

Results

The CD3 monoclonal antibody is used to determine the total T-cell level (9, 12). While, CD4+ cell level and the proportion of CD8+ cells that express activation antigens are two of the most informative lymphocyte subset parameters for predicting clinical progression in HIV infection (13). The number of CD8+ lymphocytes in peripheral blood increases shortly after HIV seroconversion and remains high after the start of depletion of CD4+ lymphocytes (6, 12, 13). In addition CD3+ and CD8+ lymphocyte levels aid in evaluating whether low CD4+ counts are due to selective depletion or general reduction of all lymphocytes (6).

Moreover, in the case of HIV monitoring, the cell population of paramount importance is the

3. Amador JF, Ramos M, Artiles V, Suárez O, García M, Taquechel N *et al.* Immunologic monitoring of renal allografted patients treated with the IOR T3 (anti CD3) monoclonal antibody. Effects on CD markers and its pharmacokinetics. Immunología 1994;13:35-36.

4. Landay A, Ho JL, Hom D, Russell T, Zwerner R, Minuty JG *et al.* A rapid method for CD4+ T-cell quantification for use in developing countries. AIDS 1993;7:1565-1568.

5. CDC, 1992. Revised classification system for HIV infection and expanded AIDS surveillance case definition for adolescents and adults (1992). U.S. Department of Health Services, Public Health Center for Disease Control, Atlanta, Georgia: report based upon CDC workshop convened April 17-18, 1990, in Atlanta, Georgia.

6. Denny T, Jensen BD, Gavin E, Lousao SAG, Vela FA, Oleske JM *et al.* Determination of CD4 and CD8 lymphocyte subsets by a new alternative fluorescence immunoassay. Clin Diag Lablmmunol 1995;2:330-336.

7. Biberfeld G. The need for alternative CD4 T-cell monitoring methods. Proceedings of the satellite symposium held in conjunction with the international conference on AIDS, Berlin, Germany, 1993;4.

8. Denny T, Yogev R, Gelman R, Skuza C, Oleska J, Chadwick E *et al.* Lymphocyte subsets in healthy children during the first five years of life. JAMA 1992; 267:1484-1488.

9. CDC, 1994. Revised guidelines for the performance of CD4+ T-cell determinations in persons with human immunodiciency virus (HIV) infection. MNWR 1994,43:1-21.

10. Rivero R, Bello M, Suárez L, Cruz C, Martínez M, Palma L. Introducción de un ultramicrométodo para la cuantificación de subpoblaciones linfocitarias identificadas con anticuerpos monodonales. Rev Cub de Hematología Inmunología y Hemoterapia 1995;1:46-56.

11. Suárez L, Cruz C, Rivero R. Ultramicrométodo inmunohistoquímico. Titulación de anticuerpos monodonales utilizados para el inmunofenotipaje celular. Rev Cub de Hematología Inmunología y Hemoterapia 1995;1:57-62.

12. Giorgi JV, Hultin LE. Lymphocyte subset alterations and immunophenotyping by flow cytometry in HIV disease. Clin Immunol Newlett 1990,10:55.

13. Landay A, Ohlsson-Wilhelm B, Giorgi JV. Application of flow cytometry to the study of HIV infection. AIDS 1990;4:479-497.

14. Landay A. Putting alternative CD4 T-cell enumeration methodologies into perspective. Proceedings of the satellite symposium held in conjunction with the international conference on AIDS, Berlin, Germany, 1993;5.

CD4+ T lymphocyte (14). Measures of CD4+ lymphocytes are currently used to guide clinical and/or therapeutic actions for HIV-infected persons (1-9, 12-14). Depletion of CD4+ T-cells is associated with increased clinical complications and is a measure of immunodeficiency. Among persons with HIV infection, CD4+ T lymphocyte determinations are used in clinical decisions for prognosis and therapy because they have been found to be useful for predicting the onset of opportunistic diseases. These determinations are also used as a surrogate for therapy outcome. In addition, persons with CD4+ T-cell levels < 200 cell/μL, or 14 % are now classified as having acquired the immunodeficiency syndrome (AIDS) using CDC's reversed classification system (9).

Thus, although all three markers (CD3, CD4 and CD8) were evaluated in the group of patients studied, in this paper, the statistical analysis was focused on the CD4+ T-cell marker.

Evaluation of CD4+ T-cell marker in the 60 patient samples studied showed a correlation of 0.95 between the standard flow cytometry method and the Opti-CIM assay (Figure 1, Table 1). When evaluating HIV seropositive samples there was a significant positive correlation (r = 0.941) between flow cytometry and the Opti-CIM assay.

Data obtained was also evaluated based on CD4+ T-cell percentages as determined by flow cytometry. This analysis demonstrated that the Opti-CIM assay was 96 % sensitive and 94 % specific. In the sample of 60 individuals studied, the Opti-CIM assay had a 97 % predictive value for correctly identifying individuals with CD4+ T-cell percentages >14 % and 93 % predictive value for correctly identifying individuals with CD4+ T-cell percentages <14 % (Table 2).

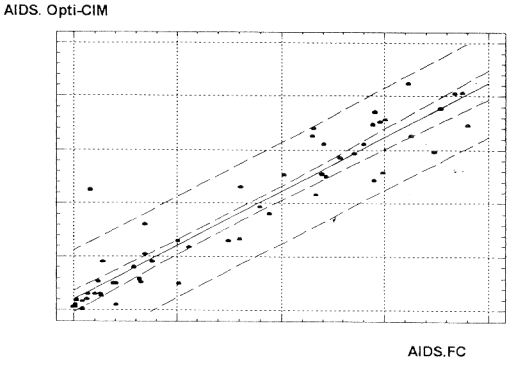

Figure 1. Scattergram CD4+ T-cell percentages in 60 individuals as determined by Opti-CIM and flow cytometry (FC).

Table 2. Sensitivity and specificity of the Opti-CIM assay.

	Opti-CIM, CD4+ T-cells		
Flow cytometry, CD4+ T-cells	≤ 14 %	> 14 %	Total
≤ 14 %	25	1	26
> 14 %	2	32	34
Total	27	33	60

Total samples: 60.
HIV seropositive: 54.
HIV seronegative: 06.
Sensitivity: 96 %.
Specificity: 94 %.
Opti-CIM has a 97 % predictive value for correctly identifying individuals with CD4+ T-cell percentages > 14 % and a 93 % predictive value for correctly identifying individuals with CD4+ T-cell percentages < 14 %.

CD4+ T lymphocytes can be easily identified by their reddish membrane immunostaining among the negatively stained T-cells in the same field (Figures 2 and 3). The green staining of the nucleus facilitates the easy identification and counting of positive and negative cells. Additionally, cell morphology and retrospective studies can be performed, if necessary, to aid in diagnosis and evaluation of prognosis.

Discussion

The Opti-CIM diagnostic kit is based on a simple and economic method for determining CD3+, CD4+ and CD8+ lymphocyte subsets. The modification developed at the Center of Molecular Immunology (CIM) (manuscript in preparation) of a previously described method (10, 11) provided a faster, simpler to perform, reproducible and accurate procedure that allows the introduction of this manual diagnostic kit, that can be used anywhere in the world. A method based on MAb coated beads, with similar advantages (4), is limited to CD4+ T-cell evaluation and does not allow the visualization of cell morphology.

The results of the CD4+ T-cell percentages compared favorably with standard flow cytometry with an overall correlation coefficient of 0.95 in a total of 60 samples evaluated. Furthermore, the Opti-CIM assay has a positive predictive value of 93 % for

Table 1. Correlation between the standard flow cytometric method and the Opti-CIM assay.

		Mean CD4+ T-cell percentages	
CD4+ T-cell Stratification	Correlation (r)	Flow cytometry	Opti-CIM
Total, n = 60	0.95	18.54 ± 14.78	21.13 ± 16.08
HIV+, n = 54	0.941	15.81 ± 12.68	17.98 ± 13.63

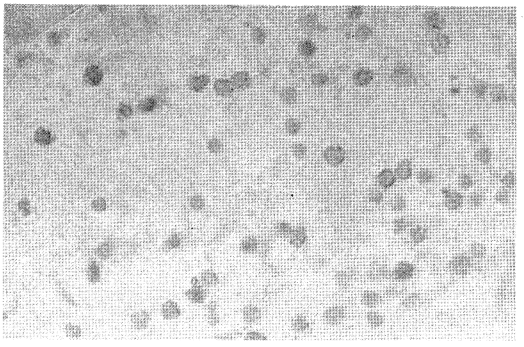

Figure 2. CD4+ T-cells in HIV negative donors. (See color plate on page 217).

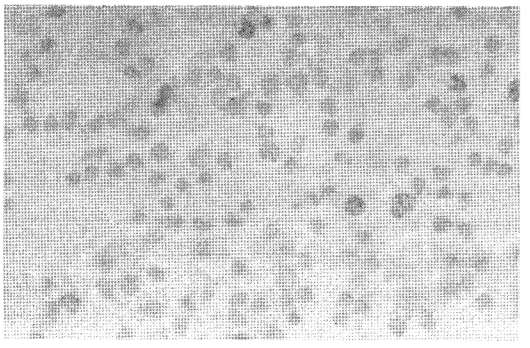

Figure 3. CD4+ T-cells in HIV (+) patients. (See color plate on page 217).

identifying individuals in the sample studied with <14 % CD4+ T-cells as the threshold for comparing the two assay methods. However the reliability and the reproducibility of the Opti-CIM assay requires further evaluation in larger longitudinal studies of individuals with HIV/AIDS.

Although treatment is currently limited to AIDS-defining illnesses, such as tuberculosis, wasting syndrome, chronic diarrhea or *Pneumocystis carinii pneumonia* in HIV infected individuals in developing countries, the monitoring of CD4+ T-cell levels may provide important information to establish the clinical stage of the disease, predict progression to AIDS and stratify HIV seropositive individuals into various treatment protocols and vaccine trials (4).

The Opti-CIM Diagnostic Kit requires only one week of training for the average technologist and only a light microscope. The cost of the Opti-CIM assay per CD3, CD4 and CD8 % determinations is approximately US$ 1.50. In contrast, CD4 % determination by flow cytometry is of US$ 35.00 per test excluding additional cost of purchasing and maintaining a flow cytometer.

In summary, the Opti-CIM Diagnostic Kit appears to be simple, reliable and less expensive and may have potential application for clinical drug and vaccine trials and staging of HIV disease and AIDS in third world countries where HIV prevalence is high and funds for flow cytometry are limited.

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