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CD4 quantification based on magneto ELISA for AIDS diagnosis in low resource settings

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ABSTRACT

The Acquired Immune Deficiency Syndrome (AIDS) affects the life of millions of people around the world. Although rapid and low cost screening tests are widely available for the diagnosis of HIV infection, the count of CD4+ T lymphocytes remains a drawback in the areas mostly affected by the HIV, being this control imperative for assessing the deterioration of the immunological system and the progression towards AIDS, when the counting of cells falls down 200 cells μL^{-1}. This paper describes a high-throughput, simple and rapid method for CD4+ T lymphocytes quantification, directly in whole blood, based on a magneto ELISA. The CD4 cells are separated and preconcentrated from whole blood in magnetic particles, and labeled with an enzyme for the optical readout performed with a standard microplate reader. The magneto ELISA is able to reach the whole CD4 counting range of medical interest, being the limit of detection as low as 50 CD4+ cells per µL of whole blood, without any pretreatment. This method is a highly suitable alternative diagnostic tool for the expensive flow cytometry at the community and primary care level, providing a sensitive method but by using instrumentation widely available in low-resource settings laboratories and requiring low-maintenance, as is the case of a microplate reader operated by filters.

1. Introduction

The induction of progressive CD4+ T cell depletion is a hallmark of the Human Immunodeficiency Virus (HIV) infection [1]. Once the HIV enters the human body, the external envelope glycoprotein gp120 interacts with the CD4 receptor to initiate the infection [2], being thus the CD4+ T lymphocytes and monocytes/macrophage lineage the predominant target cells of the virus [3]. The immune system starts producing anti-HIV antibodies (seroconversion stage) and the number of CD4+ lymphocytes in blood recovers the normal value (ranging from 500 to 1200 cells μL^{-1}) due to proliferation of cells in lymph nodes. In the next stage (clinical latency) the count of CD4+ lymphocytes is progressively depleted by factors that include the direct effects of HIV on infected cells. The duration of this clinical latency stage varies considerably from person to person, from 2 to over 10 years. Progression to Acquired Immune Deficiency Syndrome (AIDS) occurs as a result of chronic depletion of CD4 cells falling below 200 cells μL^{-1}, at a functional level where opportunistic infections and malignancies cannot be controlled [4]. The CD4+ T lymphocyte count is one of the most important clinical biomarkers of HIV disease progression, especially during the clinical latency. The count is useful for assessing the degree of immune deterioration and speed of progression towards AIDS, as well as for monitoring the efficacy of a treatment. This parameter is also used to decide the timing for starting the antiretroviral treatment (ART) and the prophylaxis against opportunistic infections. The gold standard method for CD4 count is the flow cytometry, performed in expensive instruments which are rather impractical and difficult to sustain in low-resource settings in the developing world. For instance, in Malawi in mid-2010, from a total of 396 ART delivery sites, as low as 1 in 10 sites had a functioning CD4 cytometer [5]. Moreover, in low-income countries, a health worker in a primary care centers who wish to perform a CD4 test faces not only the drawbacks in blood specimen transportation to a centralized laboratory, but also in returning back the CD4 result to the patient. Moreover, it was reported that nearly half of HIV-infected patients in South Africa, failed to have CD4 counts after HIV diagnosis [6], highlighting the urgent need to develop new alternatives of CD4 count in low resourse settings. Additional issues are the damage of blood specimens during transportation to the CD4 count central laboratory, machine malfunctions due to poor maintenance, and lack of reagents and funding limitations. The core of the problem is that current CD4 counting technology is too sophisticated and

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inappropriate in the context in which it is being used.

Currently, there are only few cheaper alternatives for CD4 counting commercially available [7,8], for instance, PointCareNOW™ (PointCare Technologies, Inc., USA), CyFlows miniPOC (Sysmex Partec GmbH, Germany), Guava EasyCD4 (Merck Millipore, USA) and Alere Pima™ CD4 (Alere, USA). Their main features are summarized in Table S4, electronic supplementary material. Although the accuracy and simplicity of most of these technologies are not under discussion, they still require costly equipment being thus not so suitable for resource-constrained small centers. On the other hand, the manual methods Coulter CD4 count kit (Beckman Coulter, Inc., USA) and Dynabeads® T4 Quant Kit (Thermo Fisher Scientific, Inc., USA) are limited to the number of samples analyzed per day, being inappropriate when a high sample throughput is an important requirement.

Since the introduction of the Enzyme-linked immunosorbent assays (ELISA) in 1971, [9,10] this methodology is widely used in clinical diagnosis in small centers due to its high sensitivity, specificity and accuracy. This methodology is recommended by the WHO for the detection of several infectious diseases affecting global heath in the developing world, including Influenza [11], HIV [12], Chagas [13], Ebola [14] among many others since it is a sensitive method but by using instrumentation available in low-resource settings laboratories and requiring low-maintenance, as is the case of a microplate reader operated by filters. Another important advantage of ELISA includes the high-throughput. As it is performed in 96-wells microplaques, a technician can analyze over 230 samples per day. Moreover, the application to novel targets is not an issue since the instrumentation is widely available in small centers.

Novel development in clinical diagnosis that is also needed involves preconcentration procedures on solid supports which can be easily integrated with point of care devices. Biomarkers in complex clinical samples can be thus preconcentrated while the interfering matrix is removed at the same time, increasing the sensitivity and the specificity of the test. One of the most prominent materials to meet this challenge is magnetic particles (MPs) [15]. MPs can be tailored to specifically bind the biomarkers and concentrate them from the complex specimen under magnetic actuation, avoiding interference before testing [16]. They are synthesized containing a magnetic element in their core such as iron, nickel, neodymium or magnetite. Nowadays several companies offer a wide range of products based on MPs, such as Adembeads, Dynabeads, BioMag, SiMAG, MACS, among many others. Recent advances have focused on their use in magneto-actuated rapid diagnostic tests (RDTs) [17]. The integration of MPs can thus simplify the analytical procedure, avoiding the use of classical centrifugation or chromatography separation strategies, since no pre-enrichment, purification or pretreatment steps, which are normally used in standard analytical methods, are required. Moreover, their use as solid support in bioassays has been shown to greatly improve the performance of the biological reactions. In this paper, a high-throughput and sensitive magneto-actuated ELISA for CD4+ T cell quantification is presented for their implementation in decentralized small care centers as an alternative to the costly standard flow cytometry. In this approach, magnetic particles were integrated in a classical ELISA format. The integration of the magnetic particles allowed the preconcentration of CD4 cells from whole blood, without any pretreatment, by immunomagnetic separation. The captured CD4+ T cells were at the same time labeled with a biotinylated antiCD4 antibody, followed by the reaction with an enzyme for the optical readout using a standard microplate reader operated by filters. The CD4 magneto ELISA demonstrated to be a powerful tool for the screening of a large number of specimens at the community and primary care level, especially in the developing world, as well as in low-resource settings in the developed world.

2. Experimental

2.1. Instrumentation

Optical measurements were performed on a TECAN Sunrise microplate reader with Magellan v4.0 software. The data were analyzed using the Graph Prism software (GraphPad Software, San Diego, CA). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, DK). Cell count was obtained by counting in Neubauer chamber using Nikon Eclipse TS100 microscopy (Nikon Instrument, USA) and by flow cytometry using perfect count microspheres (Prod. No. CYT-PCM-100, from Cytognos SL, Spain) and analyzed in a FACSCanto cytometer (BD Bioscience, USA). The immunomagnetic separation was evaluated using Telaval 31 optical microscopy (Zeiss, Alemania). The SEM images were taken with the scanning electron microscope Zeiss Merlin (Resolution: 0.8 nm at 15 kV with EDS Detector Oxford LINCA X Max). E5000 Sputter Coater Polaron Equipment Limited metallizer and K850 Critical Point Drier Emitech (Ashford, UK) were used for sample treatment. The confocal fluorescence images were taken with the TCP-SP5 Leica Microscope, being the images processed with the Imaris X64 v.6.2.0 software (Bitplane, Switzerland).

2.2. Chemicals and biochemicals

Magnetic particles modified with antiCD3 antibody (antiCD3-MPs) (Dynabeads® CD3 Prod. No. 111-51D) were purchased from Dynal Biotech ASA (Norway). Biotinylated antiCD4 antibody (antiCD4-biotin, Prod. No. 347321), antiCD4-FITC (Prod. No. 340422), antiCD3-Alexafluor 488 (Prod. No. 557694) and antiCD3-PerCP (Prod. No. 552851) were provided by BD Bioscience and the streptavidin–horseradish peroxidase conjugate (HRP 1.11.1.7) came from Roche Diagnostics. The streptavidin labeled with cyanine 5 (Strep-Cy5) dye used in confocal microscopy was purchased from Life Technologies (Prod No. SA-1011). ELISA Substrate kit (Prod. No. 34021) was purchased from Thermo Scientific.

The CD4+ T cell clone PB100.29 was obtained by cloning the infiltrating lymphocytes from a pancreatic donor organ. The expansion method is described in *Supplementary material* [18].

All buffer solutions were prepared with milliQ water and all other reagents were in analytical reagent grade (supplied from Sigma and Merck). The composition of these solutions is described in *Supplementary material*.

2.3. SEM study of the immunomagnetic separation of CD4 cells

For the immunomagnetic separation (IMS), 1000 cells μL^{-1} were incubated with 8×10^6 beads mL^{-1} antiCD3-MPs at a 1:8 ratio for 30 min at 4 °C with shaking. A washing step with PBS 0.1% BSA was then performed. Samples were then filtered with a Nuclepore filter (0.2 μm) and fixed, as described in *Supplementary material*. CD4+ T lymphocytes attached to the antiCD3-MPs were examined by scanning electron microscope (SEM) operated at 15 kV and by energy dispersive X-ray spectroscopy detector (EDS) for elemental analysis.

2.4. Evaluation of the CD4 cells/antiCD3-MPs optimal ratio by flow cytometry and optical microscopy

The optimal ratio of the CD4 cells/antiCD3-MPs in terms of binding efficiency was evaluated by flow cytometry and optical microscopy. Flow cytometry was performed with a solution of 1000 cells μL^{-1} captured with 2×10^6, 4×10^6 and 8×10^6