Two-Site Enzyme Immunoassay of CD4 and CD8 Molecules on the Surface of T Lymphocytes from Healthy Subjects and HIV-1-Infected Patients

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A highly sensitive two-site enzyme immunoassay (Capcellia®) was developed to determine the concentration of CD4 and CD8 molecules expressed on the surface of human T lymphocytes. This assay, performed in one step (20 min), involves the specific immunocapture of T lymphocytes and reaction of the CD4 or CD8 molecules with an enzyme-labeled monoclonal antibody (mAb). The results were expressed as molar concentrations of the T-cell markers on the basis of results obtained with calibrated CD4 and CD8 standards. The assay was sensitive enough to detect 0.4 pmol/L CD4 or 0.8 pmol/L CD8, which corresponded to 20 × 10⁶ CD4⁺ or CD8⁺ T cells per liter of blood. Mean concentrations in healthy adults were 17.2 pmol/L for CD4 and 22.1 pmol/L for CD8. The CD4 concentration was <8 pmol/L in 50% of HIV-1-infected patients and in 95% of AIDS patients. Given the epitopic specificity of the mAb to CD4 we used, these values correspond to the concentration of CD4 molecules free of envelope glycoprotein (gp)120.

Indexing Terms: acquired immunodeficiency syndrome/cell surface antigens/AIDS-related complex/ELISA/leukocytes

Leukocyte surface molecules have been characterized and grouped into various clusters of differentiation (J). The detection of leukocyte differentiation antigens with labeled monoclonal antibodies (mAbs) is useful for detecting cells bearing such surface antigens; flow cytometry and quantitative microscopy are the methods of choice for enumerating these populations.1 The level of expression of surface molecules varies under common physiopathological conditions. Thus, the measurement of the expression of a functional molecule may provide a direct appreciation of the functional activity of the cell. Flow cytometry is also used to precisely quantify molecules on the cell surface (2, 3). CD4 and CD8 molecules (4, 5) are glycoproteins that characterize T lymphocytes as helpers (or inducers) of the immune response or as suppressor (or cytotoxic) cells, respectively. These molecules function in the recognition of their natural ligands, i.e., the monomorphic parts of class II and class I major histocompatibility complex (MHC) antigens, for CD4 and CD8, respectively. These molecular interactions are an essential step in antigen recognition and the triggering of the immune response. This implies that the assay of CD4 and CD8 differentiation antigens represents a method for assessing T-cell function.

We developed a two-site enzyme immunoassay (Capcellia®; Sanofi-Diagnostics Pasteur, Marnes la Coquette, France) for the determination of CD4 and CD8 expressed on the surface of human T cells immobilized by selective immunocapture. Calibration curves were prepared by using reference assay wells calibrated with known amounts of CD4 and CD8 on freeze-dried cells from a CD4⁺ and CD8⁺ T-lymphocyte leukemia cell line (6). The work presented here includes the description of the method and its validation, as well as the determination of CD4 and CD8 concentrations in the blood of healthy adults and children and of human immunodeficiency virus type 1 (HIV-1)-infected patients. T-cell CD4-dependent function is considerably reduced in HIV-1-infected patients because of the progressive loss of CD4 lymphocytes (7). We therefore assayed CD4 concentrations in HIV-1-infected patients at different stages of the infectious process until acquired immunodeficiency syndrome (AIDS) was manifested. We selected mAb F101-69 for the assay of CD4 because it recognizes an epitope [amino acid positions 42 and 43 (8)] in the Ig CDR2-like region of domain V1 of the CD4 molecule that corresponds to the binding site of the 120-kDa glycoprotein (gp120) of the HIV-1 envelope. The binding of gp120 to CD4⁺ lymphocytes thus prevents immunological detection of CD4 by this labeled antibody, and permits assay of only those CD4 molecules that are free of gp120. Analysis of blood samples from HIV-1-infected patients showed that CD4 concentrations were markedly reduced, whereas concentrations of CD8 were generally little changed, except in some patients with AIDS.

Finally, preliminary evaluations demonstrated the clinical and diagnostic usefulness of determining the concentrations of the CD4 and CD8 molecules in blood. The assay is highly sensitive and can produce significant and reliable quantitative results with samples of blood containing no more than 20 × 10⁶ CD4⁺ or CD8⁺ T cells per liter of blood.
Materials and Methods

Blood samples. Whole blood was drawn by venipuncture into tubes containing EDTA and analyzed within 24 h. The three groups of adult blood donors were: HIV-1-seronegative subjects who served as controls (n = 153); HIV-1-infected patients (n = 97) at stages II, III, or IV of AIDS-related complex (ARC) from the classification system for HIV infection of the Centers for Disease Control (9); and patients with AIDS (n = 62). In addition, we studied HIV-1-seronegative children (n = 51), HIV-1-seronegative hemophiliac children (n = 34), and HIV-1-infected hemophiliacs (n = 35) at ARC stages II, III, and IV, and with AIDS. All the hemophiliacs except three had received multiple blood transfusions before 1985. The procedures followed were in accordance with the ethical standards of the hospitals' responsible committees.

Separation of mononuclear cells. Peripheral blood mononuclear cells (PBMC) were separated from blood by centrifugation on a Ficoll-Paque gradient (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden). A mixture of 0.5 mL of blood and 1.5 mL of phosphate-buffered saline (PBS) was layered onto 1.5 mL of Ficoll-Paque in a 5-mL tube and then centrifuged at 900 g for 10 min. The ring of PBMCs was removed in its entirety in exactly 1 mL; 1 mL of PBS was added. CD4 and CD8 were assayed in 75 μL of the PBMC suspension added to each microtiter well (sample equivalent to 18.7 μL of whole blood before separation).

T cell enumeration. The number of T cells per microliter of blood was determined with an automatic counter (Hematrak; Ortho Diagnostic Systems, Raritan, NJ). Percentages of CD4+ and CD8+ T-cell subsets were determined by flow cytometry (Facstar; Becton Dickinson Immunocytometry Systems, San Jose, CA). The T-lymphocyte populations were characterized by two-color immunofluorescence with mAbs to CD4 or CD8 in conjunction with mAb to CD3 (Leu3a—phycoerythrin, mAb specific for CD4 domain V1, and Leu4—fluorescein isothiocyanate [FITC], mAb specific for CD3, purchased from Becton Dickinson; and IOT8a—phycoerythrin, mAb to CD8, from Immunotech, Luminy, Marseilles, France).

Monoclonal antibodies and conjugates. We produced murine mAbs in our laboratory by hybridoma formation (BALB/c mice, P3X63Ag8.653 myeloma). MAb's F92-3A11 (IgG1), F101-69 (IgG1), and F101-87 (IgG1) were characterized as members of the CD2, CD4, and CD8 clusters, respectively, during the evaluation performed at the 3rd International Conference on Human Leukocyte Differentiation Antigens (Oxford, UK, 1986) (10). The mAbs were isolated from ascitic fluid of BALB/c mice by affinity chromatography with Protein A—Sepharose (Pharmacia, Uppsala, Sweden). The CD4 and CD8 mAbs were coupled to horseradish peroxidase (Boehringer Mannheim, Mannheim, Germany) with the sodium periodate method (11). The conjugates were analyzed by sodium dodecyl sulfate—polyacrylamide gel electrophoresis.

Preparation of assay plates. The microtiter plates (Maxisorp U; Nunc, Roskilde, Denmark) were coated overnight at 4 °C with the mAb to CD2 (5 mg/L) in PBS. After a washing with PBS, the plates were saturated for 1 h at 20 °C with PBS containing 2 g/L bovine serum albumin (Sigma Chemical Co., St. Louis, MO). The plates were then freeze-dried and stored at 4 °C in evacuated waterproof bags. The microtiter wells were rinsed twice with PBS before the assay.

Assay. The Capocellia immunoassay was performed as a single step during which the cells were simultaneously immunocaptured and allowed to react with the enzyme-labeled second antibody (total duration, 20 min at room temperature, Fig. 1). We placed 75 μL of the PBMC suspension in the microwells to assay CD4 or CD8 and also to determine the absorbance of cells not exposed to the enzyme conjugate (cell blank). A solution (75 μL) containing the CD4- or CD8-mAb conjugate was then added to the assay wells and to the wells not containing any cells (immunoconjugate blank). Specific CD4 and CD8 absorbance was calculated by subtracting both the absorbance of the conjugate in the absence of cells and the absorbance of cells in the absence of conjugate.

After washing for 15 min, the assay plates were centrifuged (200g, 4 min), a process that greatly increased the stability of T-cell immunocapture by the CD2 mAb-coated assay wells. Excess conjugate was eliminated by several washings with PBS (5 × 200 μL/well). Peroxidase substrate (100 μL of a solution of one tablet of o-phenylenediamine (Sigma) dissolved in 10 mL of citrate buffer, 0.1 mol/L, pH 5, containing 20 μL of 300 g/kg hydrogen peroxide) was then added to each well. After incubation for 20 min in the dark, 50 μL of a 0.5 mol/L solution of H2SO4 was added to the wells. Absorbance was measured on a microtiter plate reader (Molecular Devices Corp., Menlo Park, CA) at 490 nm. Assay specificity was demonstrated by placing into the assay a 100-fold excess of either the same unlabeled antibody or a labeled antibody devoid of cell specificity. All determinations were performed in duplicate.

Standards. The Ichikawa human CD4+/CD8+ T-cell line (6) was cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 100 mL/L heat-inacti-
vated (56°C for 30 min) fetal calf serum and antibiotics (streptomycin 100 mg/L, penicillin 100 kIU/L). The cells were cultured at a density of $2.5 \times 10^7$/L for 24 h before the immunoassay to permit exponential growth. Cells were collected and different dilutions prepared for the standards. Briefly, 75 µL of each dilution was added to wells coated with the anti-CD2 mAb. After 16 min of incubation and 4 min of centrifugation, the cells were freeze-dried and kept at 4°C until use.

The number of CD4 and CD8 molecules bound to the surface of the Ichikawa cells was determined by using radiolabeled antibodies against CD4 and CD8. A constant number of cells ($3 \times 10^8$) was placed in a tube along with a mixture of $^{125}$I-labeled antibody (200 000 counts/min per tube) and increasing concentrations of the same unlabeled antibody (displacement test). After shaking for 2 h at 4°C, the cells were washed, and cell-bound radioactivity was measured in a gamma counter (1260 Multigamma; LKB, Uppsala, Sweden). The results were analyzed by linear regression with the Scatchard method to determine the concentration of CD4 and CD8 in each tube.

**Statistical analysis.** Linear regression was used to compare picomolar concentrations of CD4 and CD8 with the number of CD4$^+$ or CD8$^+$ T cells in blood (total lymphocyte count multiplied by the percentages of CD4$^+$ or CD8$^+$ T cells expressing membrane CD3). The differences between the picomolar concentrations in the blood-sample groups (healthy subjects, HIV-1-infected patients, AIDS patients) were analyzed by the Kruskal–Wallis nonparametric test.

**Results**

**Calibration curves.** Absorbance values from the microwells calibrated with known concentrations of CD4 and CD8 were used to construct the calibration curves. Each curve was constructed from three points corresponding to different CD4 or CD8 concentrations of the calibrated cell standard (Fig. 2). There was a linear relation between absorbance (490 nm) and the corresponding picomole concentrations. The limit of detection was defined as the lowest concentration that corresponded to the mean nonspecific absorbance of the conjugate +2 SD. The limit of detection under these conditions was 0.4 pmol/L for CD4 and 0.8 pmol/L for CD8.

**Immunocassay Validation**

**Separation of PBMC.** Experiments to separate the PBMC from whole blood were conducted over different centrifugation periods (5–30 min). Analysis of the PBMC suspensions thus obtained showed that cell densities and quantities of CD4 and CD8 were very similar, regardless of experimental conditions (Table 1). We subsequently decided to centrifuge all blood samples for 10 min.

**Kinetics of CD4 and CD8 labeling.** CD4 and CD8 detection kinetics were studied over different incubation periods (10–70 min). Fig. 3 gives absorbance values corresponding to the measurement of CD4 in a blood sample and also the values for two standards. Detection reached 73% and 86% after incubation for 10 and 20 min, respectively (100% = absorbance at 50 min). CD8 detection kinetics were identical to those of CD4 (results not shown). Given the similarity between the detection kinetics of the samples and standards at all reaction times between 10 and 70 min, we decided to incubate all PBMC samples with the capture mAb and the immunoconjugate for 20 min.
Validation of absorbance determinations for CD4 and CD8. The specificity of the absorbance determination for CD4 and CD8 was verified on PBMC incubated in a mixture of a specific conjugate and a 100-fold excess of unlabeled antibody with the same specificity. The presence of excess unlabeled antibody reduced the assay signal by 98%. When a conjugate specific for human cardiac troponin I (i.e., totally devoid of T cell specificity) was introduced into the assay, the absorbance values obtained were identical to those measured when the anti-CD4 or anti-CD8 conjugate was placed into assay wells without cells.

Reproducibility. Intraassay reproducibility was established by measuring each of the two markers 20 times. Interassay reproducibility was established on replicate preparations of whole blood by assaying each of the two markers 11 times in duplicate. The CVs were between 4.5% and 9% (intraassay) and between 6.3% and 9.8% (interassay) (Table 2).

Assays performed on successive dilutions of PBMC suspension. Fig. 4 gives CD4 and CD8 concentrations determined on successive dilutions of a PBMC suspension. The linear relation between absorbance and concentration shows that serum components do not interfere with the assay. The lowest significant concentration measured was 0.4 pmol/L for CD4 and 0.8 pmol/L for CD8. These values correspond to the limits of detection established with the standards. These concentration limits correspond to a blood sample containing only 19 x 10^6 CD4^+ T cells and 14 x 10^6 CD8^+ T cells per liter of blood. This is equivalent to only 356 CD4^+ T cells and 262 CD8^+ T cells in the assay well.

Measurement of CD4 on lymphocytes exposed to recombinant gp120. Gp120, found in the HIV-1 envelope, binds to CD4 at the Ig CDR2-like region of domain VI of the CD4 molecule. Several identical suspensions of PBMC (2 x 10^6 cells/L) were incubated at 4 or 37°C in the presence of recombinant (rg)gpl20 (American Bio Technologies, Cambridge, MA) to evaluate the interference caused by the viral protein on the CD4 assay. Under these conditions, the total number of CD4 molecules determined decreased as the concentration of rgp120 increased (Fig. 5). Thus, only 50% of the CD4 molecules were detected (control = 100%) after 2 h of incubation with gp120 at concentrations of 1.25 or 0.4 mg/L of culture medium at 4 or 37°C, respectively. No CD4 molecules were detected at an rgp120 concentration of 5 mg/L at 37°C after 2 h. Several other experiments confirmed that mAb F101-69 is directed against the same site on CD4 as gp120: (a) at 4°C, there was no decrease in the concentration of CD4 when the anti-CD4 conjugate formed with F101-69 was replaced with an anti-CD4 conjugate formed with BFF5, a mAb specific for an epitope not associated with the viral glycoprotein binding site [these observations were confirmed by using these two CD4 mAbs labeled with either ^125I (RIA method) or FITC and analyzed by flow cytometry (results not shown)]; (b) the recombinant HIV-1 p24 protein placed in contact with the cells at a concentration of 5 mg/L of medium did not interfere with the detection of CD4 by labeled F101-69 mAb.

CD4 concentration in blood samples. CD4 concentrations in blood of healthy HIV-1-seronegative adults ranged from 8 to 38 pmol/L (mean 17.2 ± 5.8 pmol/L, n = 153, Fig. 6). The analysis of five series of 10 blood samples from healthy donors selected at random gave mean values that were close to the values given above both in terms of CD4 concentrations (in pmol/L) and cell numbers (CD4^+ T cells/L blood), i.e., 14.2–20.9 pmol/L.

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Table 2. Reproducibility of CD4 and CD8 determinations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc, pmol/L</th>
<th>Intraassay (n = 20)</th>
<th>Interassay (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>19</td>
<td>4.5</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9.0</td>
<td>7.2</td>
</tr>
<tr>
<td>CD8</td>
<td>26</td>
<td>6.3</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

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Fig. 4. Immunoassay of CD4 (A) and CD8 (B) in serial dilutions of a PBMC suspension.
Units: T cells x 10^6/L (a); concentration, pmol/L (b).

Fig. 5. Measurement of CD4 on PBMC incubated with increasing concentrations of HIV-1 rgp120 for 2 h at 4°C (b) or at 37°C (w).
and 866–1164 × 10^6 T cells/L (Table 3). This assay, performed on 50 samples, allowed us to establish the relation between the concentration of CD4 in picomoles and cell number: a concentration of 1 pmol/L corresponded to a mean of 53.7 (SD 11) × 10^6 CD4+ T cells/L of blood. The comparison of concentrations and cell numbers determined in the different blood samples showed that the mean CD4 cell density was similar in all samples analyzed (there was only a twofold variation between the lowest and highest values).

HIV-1-infected adult patients, at stages II, III, or IV of ARC, but excluding patients with AIDS, had CD4 concentrations between 1.8 and 27 pmol/L (mean 8.9 ± 4.2 pmol/L, n = 97). Patients with AIDS had concentrations between 0.4 and 8.8 pmol/L (mean 3.4 ± 2.0 pmol/L, n = 62, Fig. 6). The differences between CD4 concentrations determined in the three groups were statistically significant with the Kruskal–Wallis nonparametric test (P <0.001). Moreover, a significant correlation was observed between CD4 concentrations and cell counts for the HIV-1-seronegative subjects and HIV-1-infected patients (r = 0.90, n = 139, Fig. 7A).

Some of the HIV-1-infected patients showed a low concentration of CD4, which, when compared with the CD4 T-cell count, indicated a significant decrease in CD4 density. In these patients, 1 pmol/L corresponded to as many as 150 × 10^6 CD4+ T cells/L, whereas it corresponded to a mean of 53.7 × 10^6 CD4+ cells/L in normal subjects and in most of the HIV-1-infected patients. Such a difference could be due to either a decreased CD4 expression in certain HIV-infected patients, or a masking of certain CD4 molecules by HIV-1 gp120, or both. Studies aimed at clarifying this point are in progress.

Samples from three groups of children, ages 2–15 years, were analyzed: healthy subjects (n = 51), HIV-1-seronegative hemophiliacs (n = 34), and HIV-1-seropositive hemophiliacs (n = 35). CD4 concentrations in the three groups ranged from 6 to 24 pmol/L (mean 12.5 pmol/L), from 5.3 to 27.0 pmol/L (mean 12.5 pmol/L), and from 0 (lower than the limit of detection) to 18.8 pmol/L (mean 3.5 pmol/L), respectively. CD4 concentrations in children with AIDS ranged from 0.5 to 3 pmol/L. In the control group of children (i.e., not infected with HIV-1), the 1 pmol/L concentration of CD4 corresponded to 94 × 10^6 CD4+ T cells/L. Comparison with the results from the adult population (1 pmol/L corresponding to 53.7 × 10^6 cells/L) indicates that the densities or level of

Table 3. Relation between CD4 or CD8 concentration and number of CD4+ or CD8+ T cells per liter of blood.

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>CD4 pmol/L</th>
<th>Cells/L* blood</th>
<th>Cells/L* per 1 pmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.4 ± 5.8</td>
<td>1184 ± 350</td>
<td>64.3</td>
</tr>
<tr>
<td>2</td>
<td>14.2 ± 3.9</td>
<td>886 ± 368</td>
<td>60.9</td>
</tr>
<tr>
<td>3</td>
<td>19.0 ± 6.8</td>
<td>1018 ± 406</td>
<td>53.5</td>
</tr>
<tr>
<td>4</td>
<td>19.6 ± 3.3</td>
<td>964 ± 433</td>
<td>49.6</td>
</tr>
<tr>
<td>5</td>
<td>20.9 ± 6.2</td>
<td>1164 ± 478</td>
<td>55.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD8 pmol/L</td>
<td>Cells/L* blood</td>
<td>Cells/L* per 1 pmol/L</td>
</tr>
<tr>
<td>1</td>
<td>19.8 ± 5.5</td>
<td>455 ± 111</td>
<td>22.9</td>
</tr>
<tr>
<td>2</td>
<td>18.7 ± 7.1</td>
<td>514 ± 223</td>
<td>27.4</td>
</tr>
<tr>
<td>3</td>
<td>22.1 ± 9.0</td>
<td>503 ± 200</td>
<td>22.7</td>
</tr>
<tr>
<td>4</td>
<td>27.3 ± 4.5</td>
<td>680 ± 183</td>
<td>24.1</td>
</tr>
<tr>
<td>5</td>
<td>21.6 ± 4.9</td>
<td>602 ± 229</td>
<td>27.8</td>
</tr>
</tbody>
</table>

* × 10^6.

For each experiment, 10 blood samples from healthy adults selected at random were analyzed.
expression of the CD4 molecules is generally lower in children than in adults. The correlation between CD4 concentrations and cell counts is shown in Fig. 7B (r = 0.89, n = 77).

**CD8 concentration in blood samples.** CD8 concentrations in healthy adults ranged from 7 to 76 pmol/L (mean 22.1 ± 12.7 pmol/L, n = 128, Fig. 8). Values recorded from the 50 blood samples showed the relation between CD8 concentrations in picomoles and cell density (CD8+ T cells/L of blood). Table 3 shows that 1 pmol/L CD8 corresponded on average to 25.0 (± 2.4) x 10⁶ CD8+ T cells/L of blood. Patients infected with HIV-1 (excluding patients with full-blown AIDS) had CD8 concentrations ranging from 3.3 to 45 pmol/L (mean 20.6 ± 10.4 pmol/L, n = 80). Patients with AIDS had CD8 concentrations ranging from 2.5 to 36 pmol/L (mean 12.6 ± 8.8 pmol/L, n = 58, Figure 8). The difference between CD8 concentrations in the control and AIDS groups was statistically significant with the Kruskal–Wallis test (P < 0.005).

When the same three groups of children (ages 2–15 years) were studied, the mean values for CD8 concentrations in the three sets of blood samples were not statistically different: 14.2 pmol/L, 15.8 pmol/L, and 16.9 pmol/L, respectively. Mean values of CD8 concentrations were also lower in children than in adults (14.2 vs 22.1 pmol/L in the adults). The 1 pmol/L concentration of CD8 corresponded on the average to 32 x 10⁶ CD8+ T cells/L in children.

**Discussion**

We have presented a sensitive and rapid immunometric assay (Capcella) for the determination of CD4 and CD8 molecules expressed on the surface of human T lymphocytes. This immunoassay was used to determine the concentrations of these two markers in blood samples from adults and children who were either healthy or were infected with HIV-1 and were at different stages of the disease.

The immobilization of all CD4+ and CD8+ T cells on microtiter plates is an essential step for the exhaustive assay of the CD4 and CD8 molecules on the cell surface; indeed, assays in which cells were not immobilized were unreliable because of random losses of cells during the wash steps. Previous immunoassays for the determination of leukocyte surface molecules have included different methods for binding the cells to the plastic. Poly-L-lysine, fixed by adsorption to the microtiter wells, has been used in association with glutaraldehyde (12, 13); other agents, such as glutaraldehyde alone (14–16) and methanol, have also been proposed (17). However, the chemical fixation of the cells can lead either to false-positive reactions or to partial denaturation or loss of the molecules to be measured (18, 19). Experiments performed to bind lymphocytes with a solution of 10 mL/L glutaraldehyde resulted in the complete loss of immunological recognition of CD4 and CD8 by the corresponding specific conjugates (results not shown).

The use of polyclonal antibodies to bind cells to the plastic has also been proposed (14). However, this method of cell immobilization could mask a fraction of the molecules to be measured. In addition, it does not permit a single population of leukocytes to be selected from a mixture. We therefore evaluated and validated a method of T cell immobilization by using a mAb with CD2 specificity bound by physical adsorption to the assay wells. The CD2 receptor is present on T cells from HIV-seronegative patients, HIV-seropositive individuals, and AIDS patients (20). This cell immunocapture procedure has several advantages. (a) Simplicity: The method is limited to a single 3-min centrifugation step to place the cells in close contact with the antibodies, resulting in immobilization. Comparison of results from assays performed on cells in suspension and after immunocapture showed better reproducibility with the latter method, which required less manual dexterity by the operator. (b) Reliability: Because of the absence of chemical treatment, only unmodified cell-surface molecules are assayed and no intracellular molecule is accessible to the conjugates. This discrimination therefore allows access to only those functional molecules located on the surface of the cell. (c) Specificity: Only the cell population recognized by the antibody bound to the assay well is retained, whereas the cells that do not express the antigen are eliminated by washing.

To obtain an assay that could be performed as rapidly as possible, we also improved the conditions to separate mononuclear cells on the Ficoll-Paque gradient (sample dilution and duration of centrifugation). In this way, we obtained PBMC suspensions that were identical in regards to CD4 and CD8 concentrations and cell densities after centrifugation for 5–30 min. The presence of Ficoll-Paque (250 mL/L) in the assay did not interfere with either labeling or cell capture. Thus, we were able to introduce the mononuclear cell suspension into the wells unwashed and avoid any cell loss. The selection of mAbs with a high equilibrium affinity constant (K > 10¹⁰ L/mol) provided for short assays (20 min).

Cell immunoassays previously described for leukocyte
surface antigens were calibrated with suspensions of freeze-dried membranes (17–21), reference cells introduced into the assay wells, or suspensions of beads to bind the enzyme conjugates (22). These methods provide relative quantitative values by comparing the absorbance values simultaneously in standards and in test samples. Here we have put forward the first immunoassay that determines the absolute number of molecules on the cell surface by using standards that have been calibrated beforehand with the Scatchard determination (23). The results were expressed in picomolar concentrations. We used high-affinity enzyme conjugates to bring the limits of detection for CD4 and CD8 in the assay wells to $10^{-16}$ mol (100 amol). This sensitivity is comparable with the best performance by immunoradiometric assays for isolated molecules.

The study of blood samples from adults ($n = 153$) and from children ($n = 51$) allowed us to establish a range of values for CD4 and CD8 concentrations determined in HIV-1-seronegative subjects. These values in adults ranged from 8 to 38 pmol/L for CD4 and from 7 to 76 pmol/L for CD8. In children they ranged from 6 to 24 pmol/L for CD4 and from 4 to 30 pmol/L for CD8. The corresponding cell densities (cells/L) varied over a similar range. The level of cellular expression of the CD4 and CD8 molecules (i.e., mean antigen density per cell) could be calculated from the picomolar concentrations and cell enumeration. These analyses showed that: (a) there were only slight differences between antigen densities in all the samples analyzed (twofold variations); (b) CD4 and CD8 antigen densities were apparently lower in children than in adults.

HIV-1-infected adult patients had CD4 concentrations that were significantly reduced: $\sim 50\%$ of patients at stages II, III, and IV (ARC), and close to $95\%$ of AIDS patients had CD4 concentrations <8 pmol/L (the threshold value for CD4 in the control group corresponding to $400 \times 10^6$ CD4$^+$ T cells per liter of blood). Reductions in CD4 concentrations were also noted in HIV-1-infected children. Mean concentrations of CD8 were identical in the control group and in HIV-1-infected adults in ARC stages, and reduced by $43\%$ in AIDS patients. Several reasons could explain this considerable reduction in CD4 concentrations in HIV-1-infected patients: (a) a progressive decrease in the absolute number of CD4$^+$ T lymphocytes, a characteristic of HIV-1 infection (7); (b) the masking of certain epitopes on the CD4 molecule by complexes formed between gp120 and anti-gp120 immunglobulins (24, 25). The association of human C3d complement peptide to immunglobulins has also been described (25). These molecular complexes bound to the CD4 could also modulate its expression in a cell at the ARC stage or with AIDS; (c) down-regulation of cell-surface CD4 in cells expressing the nef gene (26).

We have demonstrated the specific effect of HIV-1 rgp120, which blocks the detection of CD4 molecules by the immunoassay. These results are in accordance with studies that showed that the F101-69 mAb used in the assay of CD4 recognizes an epitope near the amino acids in positions 42 and 43 in domain V1 of the CD4 molecule (8), and that the gp120 site is localized to a region of amino acids in position 42–49 in domain V1 (37). Thus, the concentrations of CD4 determined by our immunometric method corresponded to only those molecules not complexed with gp120, whereas CD4 molecules masked by gp120 were excluded from the measurement. It has been suggested that the binding of soluble gp120 produced in HIV-1-infected patients could form complexes with CD4 and interfere with the functional relation between CD4 and class II MHC molecules (28, 29). It was therefore logical to measure only the concentration of CD4 molecules with intact MHC recognition capacity.

Recent determinations with our assay in several patients receiving anti-retroviral treatment with azidothymidine (AZT) have shown increased CD4 concentrations (+100% to 300%) without changes of CD8 concentrations within 10 days of the start of AZT treatment (Berthier AM, et al., in preparation). These modifications were not accompanied by an increase in the number of CD4$^+$ or CD8$^+$ T cells. One explanation for this is the "unmasking" of CD4 molecules hitherto encumbered by HIV-1 gp120. Moreover, this treatment-associated increase of CD4 concentrations suggests a decrease in the absolute amounts of virus in plasma. A rapid effect of AZT treatment on circulating virus concentrations was recently observed with quantitative polymerase chain reaction (30). Finally, the increase in the surface density of functional CD4 molecules demonstrates the efficacy of AZT treatment; this cannot be shown by the enumeration of CD4$^+$ cells with flow cytometry, in which a cell is determined as positive regardless of the intensity of the immunofluorescence. The enhanced functional expression of CD4 appears to accompany an improvement in the patients' clinical condition.

In summary, the Capcellia immunoassay is adapted to the quantitative determination of CD4 and CD8 molecules and analyzes blood samples without elaborate and costly investment. The measurement of molecular concentrations is a novel method for appreciating the function of T cells and is perfectly complementary with cell enumeration. This study has defined the range and mean values for blood concentrations of T cell-associated CD4 and CD8 molecules in adults and children. Threshold CD4 concentrations for functional immune deficiency were found to be 8 and 6 pmol/L in adults and children, respectively. The high sensitivity of the assay and its measurement of gp120-free CD4 molecules render it particularly well adapted to monitoring HIV-1 infection, from all stages of ARC to full-blown AIDS.

Other potential uses for this immunoassay are analysis of T-cell marker reexpression after therapeutic depletion and hematopoietic bone marrow transplantation; follow-up studies on transplant recipients; and evaluation of patients with immune system disorders such as autoimmune disease, congenital immune deficiency, and idiopathic CD4$^+$ T lymphocytopenia (31).
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References