Quantitative Determination of CD4/CD8 Molecules by a Cell Marker ELISA
Lutz Franke, Elsa Nugel, Wolf-Dietrich Döcke, and Tomas Porstmann

Determination of percentages of CD4+ and CD8+ T cells from patients with human immunodeficiency virus infection is usually done by flow cytometric analysis. We compared a cell marker ELISA with flow cytometry for quantitation of CD4 and CD8 molecules on T lymphocytes, and correlated the values both with the number of CD4+ and CD8+ T lymphocytes and with clinical data. Results by cell marker ELISA (y) correlated well with those by flow cytometric analysis (x); r = 0.69, P < 0.001 (y = 0.01x + 3.9) for CD4; r = 0.81, P < 0.001 (y = 0.03x + 5.4) for CD8; n = 343. The ELISA detected changes in numbers of CD8 molecules on the cells earlier than flow cytometry recognized changes in CD8+ T-cell counts. The advantages of the ELISA are the small sample volume required (0.5 mL of blood), its internal standardization by a CD4+/CD8+ cell line, and its simple and fast performance. The cell marker ELISA appears to be an efficient alternative to flow cytometry.

Indexing Terms: enzyme immunoassay/flow cytometry/lymphocytes/HIV/cytomegalovirus

In human immunodeficiency virus (HIV) infection, both the CD4+/CD8+ T-cell ratio and the absolute number of CD4+ T lymphocytes are important for prognosis, classification of state of disease, treatment decisions, and monitoring of therapy (1-3). For other infections, autoimmune diseases, or after organ transplantations, cell marker analyses are essential. The functions of cell membrane molecules and the consequences of their quantitative changes in several disorders (e.g., septicemia, burns, autoimmune diseases, graft rejection) are better understood because of advances in techniques in molecular biology and in the ability to assess the state of the immune system and to give more accurate prognoses (4-8).

Cell membrane markers are usually assayed by flow cytometry. Standardization of tests for clinical application is difficult, especially when measuring quantitative cellular fluorescence intensity, because different flow cytometers differ in sensitivity. There are, thus far, no general standards; however, several standardization methods have been developed (9,10), although they are not widespread in routine diagnostic laboratories. Because the tests are only reliable when made in succession, determination always has to be done in the same laboratory. Moreover, flow cytometric analyses require expensive investments, whereas an ELISA reader is standard equipment in medical laboratories.

A reliable determination of the amount of CD4+ T cells, of the CD4/CD8 T cell ratio, and of other markers for cellular activation with ELISA is desirable. Therefore an enzyme immunoassay, Capcella™ CD4/CD8, for the quantitative determination of lymphocyte membrane markers CD4 and CD8, has been developed by Sanofi-Diagnostics Pasteur (Marne la Coquette, France). We compared this cell marker ELISA with flow cytometric analysis under routine conditions. We also performed follow-up studies of 49 patients and compared data from both methods with clinical assessment of the patients.

Materials and Methods

Patients. We obtained blood by venipuncture from 25 healthy control subjects (blood donors and laboratory staff) and from 38 HIV-infected subjects, 100 allograft recipients, and 19 patients with septicemia. All procedures were in accordance with the ethical standards of the Charité Hospital ethics committee. Potassium EDTA or sodium citrate were used as anticoagulants in the Monovette ready-for-use blood-collection systems (Sarstedt, Nümbrecht, Germany). The ages of the subjects (female and male) ranged from 6 to 64 years.

Flow cytometric analysis. We isolated mononuclear cells (MNCs) from 10 mL of citrated blood by density-gradient centrifugation with Ficoll-Paque® (Pharmacia LKB, Uppsala, Sweden). The cells were washed in phosphate-buffered saline (Biochrom KG, Berlin, Germany) and resuspended in 1.0 mL of phosphate-buffered saline containing 20 mL/L fetal calf serum (Biochrom KG). We incubated 50 µL of this cell suspension with 20 µL of the labeled monoclonal antibodies, diluted 10-fold, for 20 min at 4°C. For cell labeling, murine anti-CD4-fluorescein isothiocyanate (FITC) and anti-CD8-phycocerythrin (PE) conjugates (Becton Dickinson, Heidelberg, Germany) were used simultaneously. Unstained cells were not analyzed, but isotype-matched IgG–FITC and IgG–PE control conjugates (Becton Dickinson) were used as negative controls.

Cells from HIV-infected individuals were labeled and then fixed with 10 g/L paraformaldehyde (Serva, Heidelberg, Germany) for 7 min at room temperature (II). Measurement was made with a FACScan (Becton Dickinson) instrument standardized with the AutoComp pro-
gram and CaliBRITE™ beads (Becton Dickinson). Optical stability was tested daily with Quantum™ 24 and 25 beads (Becton Dickinson). In cases of heavy contamination of MNCs by erythrocytes, erythrocytes were lysed with FACS Lysing Solution (Becton Dickinson) for 3 min at room temperature, because lymphocytes and erythrocytes partially overlap in the scattergram. Lysis of peripheral blood MNCs altered the lymphocytic forward-angle light scatter signal markedly (12). However, these alterations did not influence the quality of lymphocyte gating and the fluorescence signals.

Lymphocytes were gated in forward-angle light scatter and right-angle light scatter. Lymphocyte gating was verified by using Leukogate™ (anti-CD45–FITC/ anti-CD14–PE; Becton Dickinson). As revealed by fluorescence backgating in the Lysis II program, >95% of all lymphocytes were analyzed in almost all cases (except in patients with septicemia). The proportion of contaminating cells in the lymphocyte gate (e.g., monocytes, erythroblasts) was ≤5% in all cases.

The percentage of CD4+ and CD8+ T lymphocytes, leukocyte numbers, and differential blood counts were used to calculate the absolute amounts of CD4+ and CD8+ T lymphocytes.

Cell marker ELISA (Capcellia CD4/CD8). To determine concentrations of CD4 and CD8 molecules on T lymphocytes, we used the enzyme immunoassay Capcellia CD4/CD8. According to the manufacturer's instructions, MNCs were obtained from 0.5 mL of EDTA–blood by density-gradient centrifugation with the separation medium (Ficoll-Paque) provided with the test kit. The MNCs were collected in 1.0 mL, and 1.0 mL of washing solution was added. Additional lysis of contaminating erythrocytes was not done. We incubated 75 µL of this cell suspension with 75 µL of peroxidase-labeled monoclonal antibodies (anti-CD4 or anti-CD8) in microtiter plates coated with anti-CD2 antibodies for 20 min at room temperature. Negative controls were incubated without conjugates. Lyophilized cells of the Ichikawa CD4+/CD8+ human T-cell line (13) are provided by the manufacturer at two different concentrations (low and high calibrators). Incubation was followed by a 3-min centrifugation of the microtiter plates at 200g to enhance cell binding. Unbound fractions were removed by manually washing the microtiter plates five times with a multichannel pipette. We added 100 µL of chromogen (o-phenylenediamine) to each well. After 20 min of incubation at room temperature the reaction was stopped by addition of 50 µL of 2 mol/L sulfuric acid. The absorbance was determined in a microtiter plate reader (LP 400; Pasteur Diagnostica, Freiburg, Germany) with dual-wavelength measurement (492/620 nm).

The concentrations of CD4 and CD8 molecules in the samples were determined on the basis of the calibration values (low and high). The normal values suggested are 8–35 pmol/L for CD4, 8–70 pmol/L for CD8. The conversion factor given by the manufacturer for determination of absolute cell numbers of CD4 is 50. In cases with values <8 pmol/L, one must assume that CD4+ T cells are <400 × 10^6/L, indicating a lymphopenia or an immune deficit, e.g., after HIV infection.

Influence of sample storage on CD4 and CD8 quantitation. We examined 18 samples from healthy donors to investigate how storage of blood or prepared MNCs may influence test results. CD4/CD8 quantitation was performed either immediately or 24 h after cell separation. Additionally, aliquots of the blood samples were stored for 24 h at room temperature, with cell separation and testing performed afterwards. Mean and SD cell numbers were determined for immediate CD4/CD8 quantitation and after 24 h of storage. Statistical analysis was performed with Student's t-test.

Influence of erythrocytes and hemolysis on CD4/CD8 quantitation. To determine the influence of contamination of MNCs by erythrocytes or hemolysis on CD4/CD8 quantitation, we substituted defined amounts of erythrocytes or erythrocytic lysates for MNC preparations obviously free of erythrocytes before performing the Capcellia assay. Erythrocytic lysates were obtained by freezing and thawing erythrocytes three times and centrifuging at 90 000g for 30 min to separate cell debris.

Determination of conversion factors for CD4 and CD8. According to the manufacturer's instructions, the concentration (pmol/L) of CD4 can be converted to CD4+ T-cell number × 10^6/L by multiplying by 50. No specifications are provided regarding determination of CD8+ T-cell numbers.

We determined the CD4+ and CD8+ T-cell numbers and the concentrations of CD4 and CD8 in 343 blood samples from all patients and from the control group by flow cytometry and by the Capcellia assay to establish the conversion factors as follows: cell number × 10^6/pmol. Samples without detectable CD4 (n = 3) or CD8 (n = 7), either by cytofluorometry or Capcellia, were omitted.

Precision (CV) of flow cytometric analysis and Capcellia with clinical assessment. The correlation coefficient, r, of both methods for CD4+/CD8+ T lymphocytes and CD4/CD8 molecules was established for all samples by linear regression analysis. Statistical significance was determined by Student's t-test.

To correlate both methods with clinical assessment, we conducted follow-up studies on graft recipients. The reasons for immunological activation (rejection, cytomegalovirus infection) were clarified by immunocytochemical examinations of fine-needle biopsies from grafted organs for infiltration by lymphocytes and cytomegalovirus antigen (CMV-Ag).

Results

Influence of sample storage on CD4/CD8 quantitation. For both methods, results were most favorable when
MNCs were collected and the tests performed within a few hours after obtaining blood samples. When whole blood was stored for 24 h at room temperature, 11 of 18 samples showed visible contamination of the prepared MNCs by erythrocytes. For flow cytometry, erythrocytolysis was necessary. Results (mean ± SD) are shown in Table 1. By Student's t-test, there is no significant difference (P < 0.001) between results in any of the groups.

Influence of erythrocytes and hemolysis on CD4/CD8 quantitation by Capcellia. Whereas hemoglobin did not influence flow cytometric analysis, erythrocyte contamination of the labeled MNCs did, requiring erythrocytolysis before measurement. Addition of as much as 10^10 erythrocytes or 200 μmol of Hb per liter had no significant influence on the results obtained by Capcellia, for either CD4 (Fig. 1) or CD8 (not shown).

Conversion factor and precision study results are shown in Tables 2 and 3. Fig. 2 shows the correlation of CD4 concentration with CD4^+ T lymphocytes as determined by cytfluorometry, and similarly for CD8.

Correlation of results with clinical assessment. We examined 49 patients at short intervals after they received organ transplants. Fig. 3 shows the CD4 values determined with both methods for one patient 15 months after liver transplantation. In this patient a chronic allograft rejection was diagnosed at the beginning of the follow-up period by biopsy. He was given an enhanced immunosuppressive therapy with 8 mg/day FK506 (Fujisawa Pharm., Osaka, Japan), a macrolide produced by Streptomyces tsukubaensis. The patient's state improved clinically and paraclinically, with a period of deterioration around day 460 reflected by activation of the im-

| Table 1. Influence of storage of MNCs and blood on Capcellia assay. |
|-------------------|-------------|-------------|
| Time of assay     | CD4         | CD8         |
| MNC, immediately  | 13.4 ± 5.1  | 25.1 ± 10.2 |
| Blood, after 24 h | 12.2 ± 4.2  | 21.6 ± 10.4 |
| MNC, after 24 h   | 12.2 ± 5.1  | 20.9 ± 8.2  |

Fig. 1. Influence of increasing quantities of hemolysate and erythrocytes on CD4 quantitation in Capcellia assay. —, addition of hemolysate; ——, addition of erythrocytes.

| Table 2. Conversion factors (CF) for CD4 and CD8. |
|-------------------|-------------|-------------|
| Subjects          | CD4         | CD8         |
| Healthy           | 73.4 ± 25   | 29.3 ± 25   |
| Sepsis patients   | 78.6 ± 19   | 25.0 ± 19   |
| HIV-infected patients | 68.7 ± 38  | 24.3 ± 38   |
| Transplant recipients | 46.8 ± 258 | 27.0 ± 254  |

| Table 3. Determination of intraassay CV by flow cytometric analysis and Capcellia (n = 11). |
|-------------------|-------------|-------------|
| Flow cytometry    | CD4         | CD8         |
| Capcellia         | 5.58        | 7.41        |
| From one MNC preparation | 6.12    | 6.83        |
| From different MNC preparations of one blood sample | 13.60   | 11.73        |

| Fig. 2. (A) Correlation of CD4 molecules measured by Capcellia and CD4^+ T cells determined by cytfluorometry; (B) correlation of CD6 molecules measured by Capcellia and CD8^+ T cells determined by cytfluorometry. |
mune system and an increase in CD4 cell count. CD8 values revealed a similar correlation with the clinical picture (not shown). Similar results with both methods for CD4 and CD8 values were obtained in a therapy of murine monoclonal anti-CD4 antibodies in a patient undergoing a rejection crisis 25 months after kidney transplantation (not shown).

Figure 4 shows CD8 values in a heart-transplant patient with CMV infection, diagnosed by CMV-Ag detection. An increase in numbers of CD8+ T cells was determined by flow cytometry 10 days later than the increase in the concentration of CD8 molecules was detected by Capcellia. Another patient with acute CMV infection showed similar CD8 values; the increase in concentration of CD8 molecules was determined by Capcellia 1 day earlier than by flow cytometry (not shown).

Discussion

Several attempts have already been made to quantify cellular membrane molecules by ELISA (14–16). Our aim was to investigate whether the Capcellia assay, especially developed to monitor CD4/CD8 T cells in HIV-infected individuals, could be applied to other disorders as well, and whether this assay could assume a legitimate place in the clinical laboratory as the method of choice for quantitation of other membrane markers.

The CVs for repeated determinations of isolated MNCs by Capcellia and flow cytometry are nearly the same, although the CV for the Capcellia assay increases with repeated isolations of MNCs. Thus, the critical step for Capcellia is the removal of all MNCs after density-gradient centrifugation of the 0.5 mL of blood. The small blood volume necessary for this ELISA permits such investigations on neonates and infants, and allows the determination of 20 to 25 different surface molecules from one MNC preparation.

Contamination of MNCs by erythrocytes (up to 10^10/L) or hemolysis (hemoglobin up to 200 µmol/L) does not affect the test results. As in flow cytometric analysis, one should utilize the sample the same day the blood is obtained. This is generally feasible because the test duration of Capcellia does not exceed 60 min and can be performed in almost every diagnostic laboratory without additional equipment. However, because there is no statistically significant difference between results from samples determined immediately after collection and after storage for 24 h, one can perform the test the next day.

For further evaluation of Capcellia, we conducted follow-up studies of transplant recipients. These studies revealed a good correlation with flow cytometric analysis (Fig. 3). Therapy with murine monoclonal anti-CD4 antibodies had no influence on the correlation of the results from both methods.

Investigations by the manufacturer have shown that antigen density per cell may be subject to variation. The values determined for 400 × 10^6 CD4+ cells/L were 4.6–9.9 pmol/L CD4 molecules and those for 400 × 10^6 CD8+ cells/L were 8.8–17.2 pmol/L CD8 molecules (data kindly provided by Sanofi–Diagnostics Pasteur). Therefore, the factors we used to convert concentrations of CD4 molecules into cells per liter were only relative and were used as guides. It is possible that the number of receptors per cell increases with cellular activation, thus enhancing cell function before proliferation of the cell population. The direct relation between density of membrane molecules and functionality of cells was proved for CD14, HLA-DR (17), IL-2R (18), and CD8 (19).

In two patients with CMV infection, activation of CD8+ T cells was determined by Capcellia earlier than by flow cytometry for cell counts (Fig. 4). The question of whether an increase in the concentration of molecules per liter of blood really correlates better with the functional state of the examined cell population than does the absolute number of cells expressing the molecule requires further investigation. However, in CMV infection, only the CD8+ T-lymphocyte subpopulation with a high density of CD8 molecules increases, whereas T lymphocytes with a low density of CD8 molecules are not influenced (20).

For special problems, other cell marker ELISA variations or combinations of cell ELISAs for various deter-

![Fig. 3. Time course of CD4 molecules and number of CD4+ T cells in a patient after liver transplantation.](image1)

![Fig. 4. Time course of CD8 molecules and number of CD8+ T cells in a heart-transplant patient with acute CMV infection due to immunosuppressive therapy.](image2)
minations can be used. Baumgarten (21) describes a cell marker ELISA for quantitation of mitogenic T-cell activation as an example of the quantitative determination of leukocyte antigens. Thus, with a view to decentralized diagnostics, cell marker ELISAs may represent an efficient alternative to flow cytometry. However, for complex investigations of cellular activation processes of the immune system, they will not be able to replace flow cytometry, which will keep its important place in medicobiological research.

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References


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