Rapid Enumeration of T Lymphocytes by a Flow-Cytometric
Immunofluorescence Method

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Combined with current advances in microprocessor-controlled flow cytometers, monoclonal antibodies provide a rapid means of phenotyping individual cell surface markers for a large number of clinical samples accurately and reproducibly, which may provide useful information in diagnosing disease and monitoring patients. We have developed a one-step flow-cytometric immunofluorescence procedure for enumerating E-rosette lymphocytes from whole blood by using the monoclonal antibody OKT11. This antibody recognizes the sheep erythrocyte receptor on the lymphocyte surface and can block sheep E-rosette formation. The flow cytometer we used, an Ortho Spectrum III, distinguishes lymphocytes from other leukocytes by measuring the narrow forward and right-angle light-scattering properties of the cells. The instrument further differentiates T lymphocytes from non-T lymphocytes by measuring the green fluorescence signal of the OKT11-positive lymphocytes. In a typical sample, 1500–2500 lymphocytes are counted in 25 s. In a study of 158 patient samples, ranging from 1% to >90% E-rosette-positive lymphocytes, the correlation coefficient between the manual E-rosette count and the flow immunofluorescence measurement is 0.943.

Additional Keyphrases: monoclonal antibodies • immunofluorescence

Human peripheral blood lymphocytes can be divided into several subpopulations, based on their cell surface markers as well as their functional properties (1). Changes in the relative percentages of various subpopulations have had major clinical application in assessing leukemic patients and other autoimmune diseases (1–5).

Until the recent discovery of hybridization techniques for production of monoclonal antibodies, identification of T and B lymphocytes and lymphocyte subsets was technically difficult, if not impossible. The enumeration of T lymphocytes by the classical manual E-rosette method is time consuming and often requires technical experience and individual judgment. Furthermore, age and storage conditions of the sheep erythrocytes cause large experimental variations and different stabilities of the rosettes (6, 7).

Recently, various monoclonal antibodies produced against specific lymphocyte antigens have been identified and are commercially available. Many of these antibodies are proven diagnostic aids in classification of immunological diseases (8, 9).

In this study, we report a rapid, flow-cytometric immunofluorescence method1 for studying E-rosette T cells in whole blood by using fluorescein-conjugated OKT11. The method can also be used with minimum modification for studying other cell markers and to identify cells with multiple markers (10, 11).

Materials and Methods

Blood cells. Normal peripheral blood samples were obtained from healthy donors. Abnormal patients' samples were obtained from various hospitals throughout the eastern United States. The samples were collected into EDTA-containing Vacutainer Tubes (Becton Dickinson, Rutherford, NJ 07070) and processed within 24 h. The percentage of various lymphocyte subpopulations in whole blood during this storage period was the same as in freshly drawn blood.

Monoclonal antibody. OKT11 monoclonal antibody2 was produced and isolated as described by Kung et al. (12). Purified monoclonal antibody was conjugated to fluorescein isothiocyanate (FITC) according to the method of Goldman (13) with a final fluorescein/protein ratio of 5.4. FITC-conjugated OKT11 is available as a commercial product of Ortho Diagnostic Systems Inc., Raritan, NJ 08869.

Immunofluorescence method. For direct immunofluorescent staining, we added 10 μL of appropriately diluted monoclonal antibodies to 100 μL of whole blood. After 25 min of incubation on ice, we added 2 mL of lysing solution (per liter, 8.3 g of NH₄Cl, 1 g of KHCO₃, and 0.37 g of Na₂EDTA, pH 7.4) to the sample for 5–10 min at room temperature, then analyzed the sample without delay with an Ortho Spectrum III Laser Flow Cytometry System. Alternatively, for batch-wise analysis, we pelleted leukocytes by centrifuging the sample tubes at 4 °C at 250 × g for 5 min. The resulting pellet was resuspended in 1 mL of cold calcium- and magnesium-free phosphate-buffered saline containing 1 g of bovine serum albumin and 0.1 g of sodium azide per liter (PBS-BSA buffer), then analyzed with the flow instrument.

Briefly, the instrument distinguishes lymphocytes from other leukocytes in blood by measuring the right-angle and narrow forward light-scattering properties of each cell. While identifying a lymphocyte by its particular light-scattering properties, the instrument further classifies the cell by recording its green fluorescence signal. In a typical experiment, about 1500 lymphocytes were counted. The electronic identification of the different cell types was confirmed by cell-sorting experiments with an Ortho Cytofluorograf Cell Sorter.

For two-color immunofluorescence experiments with T and B cells, we first labeled buffy-coat cells with nonfluorescent monoclonal antibodies for 20 min at 4 °C. Erythrocytes were lysed with ammonium chloride lysing buffer. Leukocytes were pelleted and washed with PBS-BSA buffer. We then incubated the labeled sample with 25 μL of human AB serum and 50 μL of diluted biotin-labeled horse anti-mouse antibody (Vector Laboratories, Burlingame, CA 94010) for 30 min at 4 °C. After washing this sample twice with PBS-BSA buffer, we added 25 μL of diluted rodamine-labeled avidin (Vector Laboratories) and 25 μL of human AB serum to the resuspended cell pellets, incubated for 10 min, then added 25 μL

1 We used clone OKT11A, available as Ortho-Mune OKT11.

2 U.S. patent no. 4284412.

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of a solution of mouse IgG (1 g/L) and 10 μL of the FITC-labeled OKT11 monoclonal antibody. After 30 min of further incubation, the sample was washed once and resuspended in PBS-BSA buffer for analysis by flow cytometry. For the two-color analysis, we operated the Ortho Spectrum III Laser at 514 nm and 70 mW. An Ortho 2150 Computer was used for on-line data acquisition.

E-rosette test. We used sheep erythrocyte rosettes as T cell markers, treating sheep erythrocytes with S-(2-aminoethyl)-

isothiouronium bromide hydrobromide, according to the method of Pellegrino et al. (14). The E-rosette-forming T lymphocytes were counted manually by light microscopy. Monocytes were excluded from the count according to their morphology, after staining the cells with Toluidine Blue O (10 g/L). In a typical experiment, 500 cells were counted. Cells rosetted with three or more sheep erythrocytes were considered positive.

Other experiments. For the blocking experiment, 1 × 10⁶ mononuclear cells isolated from Ficoll–Hypaque centrifugation were incubated with OKT11 monoclonal antibody for 25 min at 4 °C at a concentration of 5–10 mg/L. The cells were washed and subsequently used for E-rosette formation.

Flow instrument. The optical arrangement of the Ortho Spectrum III is shown in Figure 1. The instrument collects four kinds of signals simultaneously: when cells pass through the laser beam in the flow channel, the instrument records the forward and right-angle light-scattering signals as well as the red and green fluorescence signal of the cells.

Results

Lymphocyte analysis. A light-scattering profile for leukocytes from a typical experiment is shown in Figure 2A. Lymphocytes can be distinguished from other leukocytes on the basis of their forward narrow-angle and right-angle light-scattering properties. The percentage of lymphocytes counted in region 1 correlates well with results by a manual leukocyte differential (r = 0.971) over the range of 2 to 99% lymphocytes (Figure 3). We also identified the light-scattering properties of lymphocytes by cell-sorting experiments. The average purity of lymphocytes sorted from more than 40 blood samples was 98%. Detailed results from the sorting experiments will be presented elsewhere.

T lymphocyte analysis. For analysis of lymphocyte subpopulation, the Ortho Spectrum III also records the fluorescence intensity signal of each individual lymphocyte and displays the results of the analysis on a histogram, as shown in Figure 2B. When lymphocytes in whole blood were labeled with fluorescein-conjugated OKT11 monoclonal antibodies, the percentage of positive lymphocytes measured by the flow-immunofluorescence method correlated well with the percentage of E-rosetted lymphocytes counted manually (r = 0.943, n = 158) over the range 1 to 90% T lymphocytes.
Fig. 3. Correlation of manual lymphocyte count vs Ortho Spectrum III lymphocyte count by selective differential analysis of same lymphocyte clusters
Ortho Spectrum III analyzed between 3000 and 4000 leukocytes; after staining with Wright's stain, 200 leukocytes from the same samples were counted manually on slides. \( y = 0.927x + 0.74 \) (\( r = 0.971 \))

(Figure 4). Precision for T cell analysis was also good: for a typical experiment, in which between 1500 and 2000 lymphocytes are counted by the instrument, the CV was generally in the range of 2 to 5% for T-cells content of 60 to 80% (Table 1).

OKT11 monoclonal antibody was tested for its ability to inhibit E-rosette formation. Incubating mononuclear cells (1 \( \times 10^6 \)) with 5 \( \mu \)g or more of OKT11 per milliliter before an E-rosette test completely blocked rosette formation. For a typical sample, 80% of the lymphocytes are identified to be positive by either E-rosette test or by OKT11 labeling. Less than 0.3% of the lymphocytes formed E-rosettes with sheep erythrocytes after incubating with OKT11 monoclonal antibody.

T and B cell analysis. Two different cell-surface antigens can be analyzed simultaneously by the two-color immunofluorescence method. The two-color method of using a single laser described here provides sufficient sensitivity for most T and B cell analyses. The fluorescent signal of human lymphocytes stained with various combinations of Ortho-Mune antibodies was measured with the Ortho Spectrum III with the laser tuned to emit light at 514 nm. As shown in Figure 5A, lymphocytes were first identified by the forward- and right-angle light-scattering cytogram. Lymphocytes in region 1 were further analyzed in a fluorescence cytogram (Figure 5, B–D). When lymphocytes were stained with two different anti-T cell monoclonal antibodies (OKT3 and OKT11), two kinds of T lymphocytes were observed in normal human peripheral blood. Most of the E-rosetting lymphocytes also bear the OKT3 antigens (OKT11\(^+\) and OKT3\(^+\)), but on the average 13% of the low-density OKT11\(^+\) lymphocytes do not have OKT3 antigens (OKT11\(^+\) and OKT3\(^-\)). Similarly, when whole-blood lymphocytes are stained with antibodies against T and B cell markers, separate clusters of lymphocyte subclasses form (Figure 5, C and D). This method can also be used to investigate expression of multiple receptors in activated T cells and abnormal lymphoid cells.

Discussion
Identification of T and B lymphocytes by using various cell surface markers is generally accepted as a useful aid in the diagnosis of leukemia and many other autoimmune diseases (1, 2). However, the current E-rosette method and the manual immunofluorescence method require relatively large quantities of blood and complex separation techniques. The manual assays are also tedious and time consuming. With the advent of cell-fusion techniques, a growing variety of monoclonal antibodies reactive to human blood cells have been described (15–19), many of which may be extremely useful in routine disease classification. The direct immunofluorescence method we describe would provide a rapid objective means of analyzing blood cells.

OKT11 appears to be a pan-T antibody defined against the sheep E rosette receptor or against a closely associated determinant, as judged from the correlation studies and the blocking experiments. Recently, Verbi et al. (19), studying OKT11 in a series of leukemic cell lines and blood samples of leukemia patients, concluded that OKT11 identifies a mature pan-T determinant. A similar monoclonal antibody (designated 9.6) that blocks the sheep E rosetting sites on lymphocytes was described by Kamoun et al. (20). Our recent study (unpublished) indicated that monoclonal antibodies.

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<th>Table 1. Precision of OKT11 Antibody Analyses for T Lymphocytes</th>
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<td><strong>T lymphocytes, %</strong></td>
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<td>20 replications in 30 min</td>
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OKT11 and 9.6 may identify different determinants on the same cell-surface macromolecule. Many clinical observations related to T-cell disease have been based on this marker, as studied with the traditional E-rosetting technique. OKT11 can be used to replace the E-rosetting test and offers many technical improvements, including speed, ease of use, and accuracy.

The fluorescence-activated cell sorter has been used for many cell surface marker studies (21); however, technical complexity of operation has made it difficult to use in a clinical setting. Use of Ortho Spectrum III, a multiparameter, semiautomated flow instrument, coupled with the power of monoclonal antibodies, makes it possible for clinical research laboratories to investigate expression of cell surface markers in various diseases.

References
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