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Human T Lymphocyte Differentiation Antigens: Effects of Blood Sample Storage on LEU Antibody Binding[1]

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Current studies of human T lymphocytes and their subsets often use quantitative immunofluorescence analysis with monoclonal antibodies against cell surface antigens. With storage of whole blood or separated mononuclear cells for more than a few hours we have found marked changes in lymphocyte analysis using a fluorescence activated cell sorter (FACS). Experiments were done to determine if these lymphocyte changes were influenced by storage temperature and if lymphocytes could be made more stable by addition of culture media RPMI 1640 to whole blood. Optimal conditions found for blood storage were with with addition of 50% RPMI 1640 and with samples held at room temperature (22° C). With these storage conditions, delay on FACS analysis up to 24 hours did not result in spurious results. When blood samples are collected in places remote from the laboratory or when batch analysis of serially collected samples is desirable, excessive storage times should be avoided.

Key terms: T lymphocytes analysis, flow cytometry methods, fluorescent antibody technique, monoclonal antibody immunology, surface antigen analysis

Many studies of human lymphocytes use quantitative immunofluorescence analysis with monoclonal antibodies against lymphocyte surface antigens and a fluorescence activated cell sorter or similar instrument, to identify B cells, T cells and subsets of T cells (2, 3). In the setting of clinical research it is often convenient to collect samples over a period of time for batch processing and sometimes to transport blood samples or separated lymphocytes to a distant laboratory for analysis. Our usual procedure has been separation of lymphocytes from the blood soon after phlebotomy and storage of the cell preparation in culture media and 5% calf serum at room temperature for only a few hours before analysis. With longer storage of whole blood or of Ficoll-Hypaque separated mononuclear cells we have found marked changes in the light scatter patterns and surface antigen staining qualities. We have studied different storage temperatures and the addition of culture media to whole blood samples to permit accurate analyses after reasonable delays.

Materials and Methods

Blood was collected from healthy volunteers into Vacutainer 4606 tubes (Becton-Dickinson, Sunnyvale, CA) containing 1.5 ml acid citrate dextrose (ACD) anticoagulant and with an approximate draw of 8.5 ml. Whole blood was stored either at room temperature (22°C) or in a refrigeration at 4°C before lymphocyte separation, antibody labeling and analysis. To test the possible protective effect of tissue culture media on lymphocytes in whole blood, RPMI 1640 (Irvine Scientific, Santa Ana, CA) was added to aliquots of anticoagulated blood in volumes equal to the blood sample. Blood samples with RPMI added were also tested for stability at 22°C and 4°C.

Mononuclear cell separation was done as follows: The whole blood was diluted 1:1 with RPMI 1640 containing no serum and layered over 10 ml Ficoll-Paque (Pharmacia Fine Chemicals, Upsalla, Sweden) in a 50-ml conical centrifuge tube. The tubes were centrifuged at room temperature for 40 min at 2000 rpm. The interface was collected and resuspended in RPMI 1640 containing 5% newborn calf serum and centrifuged 15–20 min at 1500 rpm. The pellet was resuspended, filtered through nylon mesh and counted. These cells were then stained 20 min in microtiter plates on ice by reacting 10^6 target cells/well with saturation levels of directly fluorescein-conjugated Leu antibodies (Becton Dickinson Monoclonal Center, Mountain View, CA). Cells were then washed with RPMI 1640 plus 10% fetal calf serum and analyzed immediately using a fluorescence activated cell sorter (FACS, Becton-Dickinson).

FACS analysis was done for each sample by selecting from the light scatter histogram, a population of cells corresponding to lymphocytes (1). Fluorescence intensity histograms were obtained for three monoclonal Leu antibodies (Leu 1, Leu 2a, and Leu 3a). The fraction of positive cells was determined by integrating the area under the curve of the fluorescence histogram of stained cells beyond the point where no unstained cells were found. Figure 1, *a–e*, displays light scatter and fluorescence intensity histograms of typical samples.

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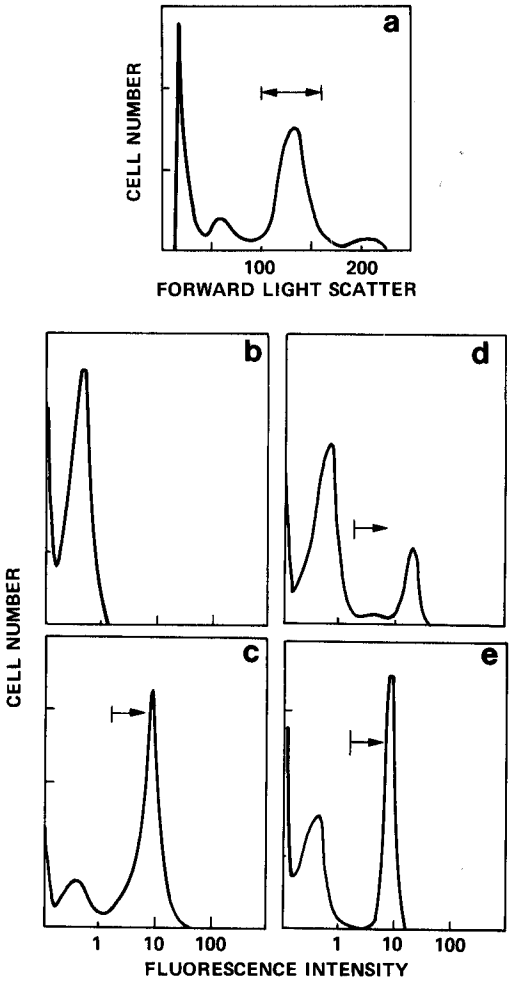

FIG. 1, *a–e*. Format of data obtained for each sample analyzed by fluorescence activated cell sorter (FACS). Panel *a* shows the forward light scatter histogram with arrows and brackets indicating the lymphocyte range. The fluorescence intensity histograms *b–e* show data for cells gated on lymphocyte light scatter and FACS analysis of unstained cells, *b*; Leu 1 positive cells, *c*; Leu 2a positive cells, *d*; Leu 3a positive cells, *e*. In each panel the single bracket and arrow show the region of the histogram integrated to determine the fraction of positive cells.

To evaluate the fluctuation in measured fractions of Leu antibody positive cells when analysis is done without delay, we performed seven parallel analyses on a fresh blood sample at ambient laboratory temperatures ranging from 18–22°C. For Leu 1 the measured fraction was 68.3% with a coefficient of variation (cv) of 2.2%. In the following discussion of results obtained with different conditions for blood sample storage, we have designated storage conditions with losses of greater than 5% as unacceptable.

Results

Mononuclear leukocyte separation with Ficoll immediately after phlebotomy and then storage of the separated cells in RPMI 1640 plus 5% serum at room temperature allowed maximum delay before analysis without causing significant change in light scatter or in the fractions of cells positive with the Leu antibodies. Analysis of cells prepared in this way immediately after phlebotomy showed no marked changes in light scatter characteristics and no changes in Leu 1, Leu 2a or Leu 3a fractions exceeding 5% of the total lymphocyte peak even when analysis was repeated up to 30 hr later. FACS analysis done at 48 hr showed changes in light scatter patterns and in some samples showed also shifts in Leu fractions exceeding 5%.

Storage of blood samples beyond 24 hr before Ficoll-Paque separation gave spurious results regardless of the storage temperature of the added media. The optimal condition tested was the addition of RPMI 1640 to fresh blood with storage at 22°C. Results with storage under these conditions are shown in Figure 2. Samples held beyond 24 hr invariably had signifi-

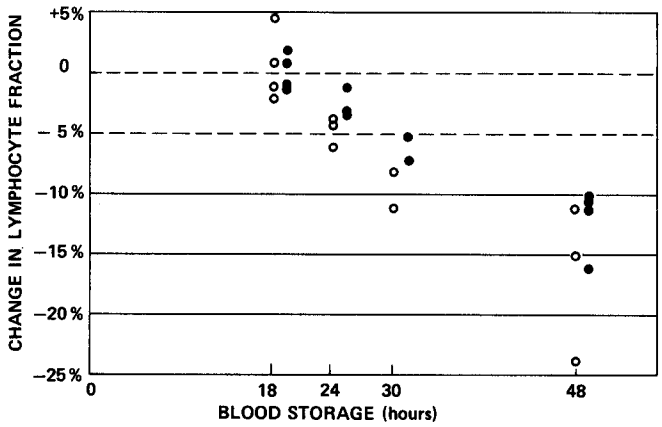

FIG. 2. Change in fraction of Leu 1 (○) and Leu 3a (●) positive cells with blood stored at 22°C in 50% RPMI. By comparison, the same blood samples stored 18 hr at 4°C had a mean decrease of the Leu 1 fraction of 8.5%. When stored 18 hr at 22°C without RPMI the mean decrease in the Leu 1 fraction was 8.0%.

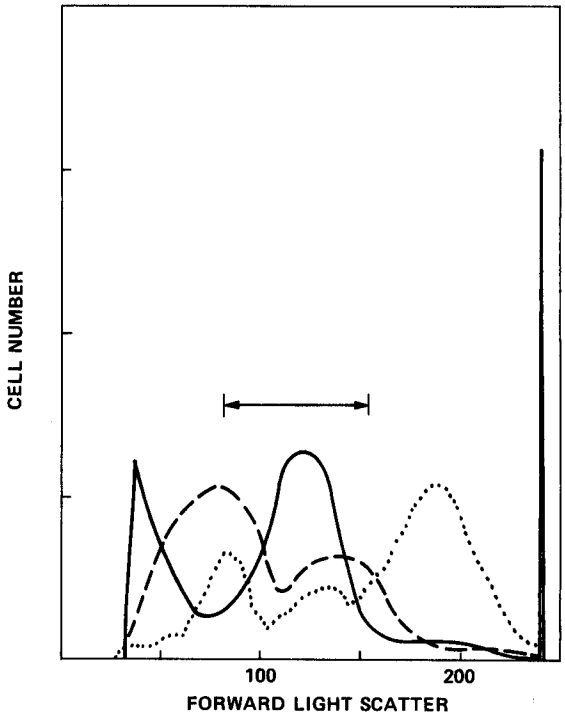

FIG. 3. FACS histograms of forward light scatter with fresh cells (——), the same cells stored for 30 hr (----) and 96 hr (····). The peak in the *solid line* under the bracket is the area normally representing the light scatter of lymphocytes.

cant losses of T cells as shown by the fractions of Leu 1 positive lymphocytes, paralleled by decreases in the Leu 3a fraction, a marker of helper T cells. When decreases in the fractions of Leu 2a and Leu 3a were expressed as fractions of Leu 1, there did not appear to be selective losses of either subset. Furthermore, for unknown reasons, the fraction of lymphocytes lost from the different subsets after blood storage was highly variable among individuals.

We found that light scatter patterns were always clearly aberrant in samples which had significant shifts in Leu fractions. Typical changes in light scatter patterns which were associated with unreliable Leu results are illustrated in Figure 3.

Discussion

The data presented here demonstrate that studies of T lymphocytes using Leu antibodies should be designed to include separation and analysis of cells as soon as possible. If a delay is required for transport of samples or for batch analysis of serially drawn samples, it is best to make lymphocyte preparations soon after phlebotomy.

When facilities for Ficoll lymphocyte separation are not readily available, the deterioration of lymphocyte morphology and staining can be slowed by addition of RPMI 1640 with storage at 22°C. The adverse effects of refrigeration and storage of the blood without added RPMI were found to be cumulative. Storage of whole blood samples for longer than 24 hr at room temperature or even shorter periods at 4°C may give spurious results. Because of marked individual variation in the loss of Leu positive cells with storage, a correction factor cannot be determined.

Careful attention should be given to the shape of the light scatter curve when selecting samples for FACS analysis. Changes from normal in the shape of the scatter histogram often indicate significant deterioration of lymphocytes which influence the fluorescence analysis.

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