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Immunology

Simple and rapid measurement of human T lymphocytes and their subclasses in peripheral blood

(flow cytometry/hybridoma/light scatter)

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ABSTRACT **A simple and rapid method for the determination of human T lymphocyte subclasses in buffy coat preparations or whole blood is described. This technique uses flow cytometry to distinguish lymphocytes from other leukocytes on the basis of their light-scattering properties. Lymphocyte subclasses were enumerated by cellular immunofluorescence; the immunofluorescent signals were produced by monoclonal antibodies to surface differentiation antigens on T cells. Conventional techniques of enumerating T lymphocyte subclasses entail time-consuming (up to 2 days) density gradient and E rosette enrichment, and require at least 20 ml of blood. The method described here uses as little as 50 μl of whole blood for each antibody tested and produces results within 2 hr.**

There is widespread consensus that the enumeration of T cells, B cells, and their functional subclasses in human peripheral blood is important in assessing immunological status in human disease (1–3). However, functional testing of T lymphocyte activities is slow, complex, and time consuming. More economical methods, such as the use of enzyme markers, the binding of erythrocytes or bacteria, and the use of antisera to cell surface differentiation antigens have been tried with some success (1, 4–6), but difficulties in standardizing reagents or in fractionating lymphocytes prior to analysis have prevented their widespread use (7).

The observation by Salzman *et al.* (8) that human lymphocytes, monocytes, and granulocytes have differing light scattering properties suggested the use of a flow cytometer to distinguish lymphocytes from other peripheral leukocyte types optically and electronically. Lymphocytes that had reacted with monoclonal antibodies could then be assigned to subclasses by cellular immunofluorescence. T cells and their subclasses could thus be enumerated within 2 hr.

MATERIALS AND METHODS

Cell Preparation and Antibody Binding. Whole blood from healthy donors 20–65 years old, was collected in Vacutainer tubes containing EDTA or heparin. The buffy coat was removed after centrifugation of the tube at 300 × *g* for 10 min; 50 μl of this cell suspension or whole blood was then incubated with 100 μl of appropriately titered monoclonal antibodies at 4°C for 30 min, treated with 2–4 ml of lysis buffer (8.29 g of NH_4Cl, 37 mg of disodium EDTA, and 1 g of $KHCO_3$ per liter, pH 7.3) for 2–5 min at room temperature. The supernate was decanted after centrifugation at 200 × *g* for 5 min. The pellet, containing leukocytes, residual platelets, and erythrocyte ghosts, was washed once in phosphate-buffered saline or RPMI-1640 (GIBCO) and incubated at 4°C for 30 min with 100 μl of fluorescein-conjugated goat anti-mouse immunoglobulin, fluorescein-to-protein ratio = 2.5–3.5 (Meloy Laboratories, Springfield, VA). After incubation and two washes with buffered saline or RPMI-1640, the cells were diluted to a final volume of 1 ml with RPMI-1640 containing 25 mM Hepes, 5% fetal calf serum, and 3 mM EDTA at pH 7.4.

The erythrocyte rosette method was used for the purification of T lymphocytes from mononuclear cells (4). The analysis of antibody reactivities of T lymphocytes was accomplished by the same procedures used for the buffy coat cells.

Reactivities of Anti-Human T Cell and Other Monoclonal Antibodies. The production and reactivities of some of our monoclonal antibodies to human T cells have been described previously: OKT1.PAN and OKT3.PAN are reactive with all peripheral T cells; OKT4.IND reacts with the inducer (helper) T cells in peripheral blood (9–11). Additional monoclonal antibodies, whose reactivities will be reported in detail, include: OKT5.SUP and OKT8.SUP, which react with peripheral T cells involved in cell-mediated cytotoxicity and suppression (12, 13); OKM1.M/G, which reacts with monocytes and granulocytes, and OKI1.FRA, which reacts with Ia-like molecules on peripheral B cells, monocytes, and a proportion of activated T cells (14, 15).

Flow Cytometric Analysis. The antibody-reactive cells were analyzed on a Cytofluorograf (FC200/4800A, Ortho Instruments, Westwood, MA) that was modified to provide simultaneous measurement of the forward light scatter, right-angle light scatter, and green fluorescein fluorescence of each cell in the sample as it passed through a laser beam (Fig. 1). In the Cytofluorograf the cells in suspension flow through a small cuvette and are individually illuminated by a narrowly focused beam from an argon 488-nm laser. The illuminated cells emit scattered 488-nm light and also fluorescent light of longer wavelengths. The right angle and the forward 488-nm scatter signals were displayed as a cytogram on the 4800A analyzer. The instrument's controls were manipulated to produce a gate pulse for cells having particular combinations of forward and right-angle light scatter intensities. The green fluorescence pulses were electronically integrated by a separate module added to the 4800A and were recorded on a pulse-height analyzer (model 2101, Ortho Instruments). The gate pulse from the 4800A was used to record fluorescence pulses from only those cells with lymphocyte light-scattering properties.

The identity of each leukocyte type displayed was confirmed by cytological examination of cells sorted with a cell sorter (Cytofluorograf 50H, Ortho Instruments).

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Abbreviation: E^+, sheep erythrocyte rosetted cells.

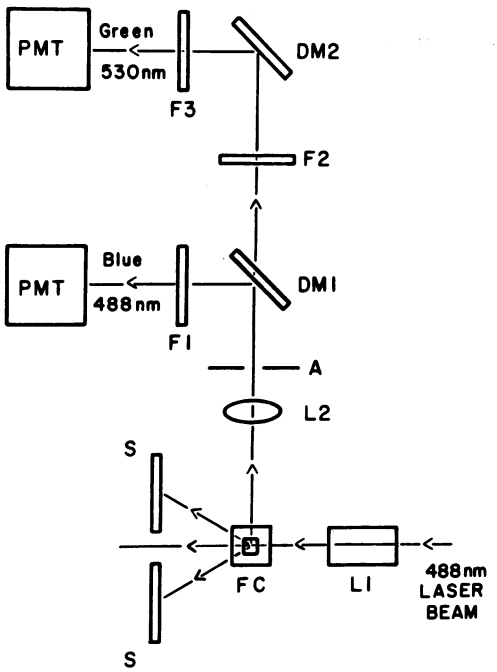

FIG. 1. Optical arrangement of the Cytofluorograf. The argon ion laser beam is focused by lens assembly L1 to an elliptical spot inside the flow cell FC. Light scattered in the forward direction is detected by silicon photo diodes S. Lens L2 collects light orthogonal to the incident beam and focuses it into aperture A. Beam splitter DM1 and filter F1 select blue scattered light. Filter F2 blocks blue light, and dichroic mirror DM2 and filter F3 select green fluorescence light. Sample flow is perpendicular to the plane of the paper. PMT, photomultiplier tubes.

Statistical Method. The simple correlation coefficient of the data obtained with the buffy coat and sheep erythrocyte rosetted cell (E^+) preparations was calculated by Pearson's correlation analysis, and the simple linear regression analysis was done by the least-squares method.

RESULTS

Light-Scatter Analysis of Peripheral Leukocytes. Analysis of buffy coat leukocytes by forward and right-angle light scatter, in agreement with the observations of Salzman *et al.* (8), showed four clusters in the cytogram (Fig. 2*A*). The number of cells in each cluster was integrated to give a simple and rapid estimate of the percentages of lymphocytes, monocytes, and granulocytes in a sample. Cells in the monocyte cluster (cluster 2) generally decreased, after the cells were stained, in relation to the other cell clusters (Fig. 2*B*). This may be attributed to the adhesion of monocytes to the glassware used during the staining procedures. The immunofluorescent staining procedure did not affect the relative forward and right-angle light scatter signals from buffy coat leukocytes (Fig. 2*B*).

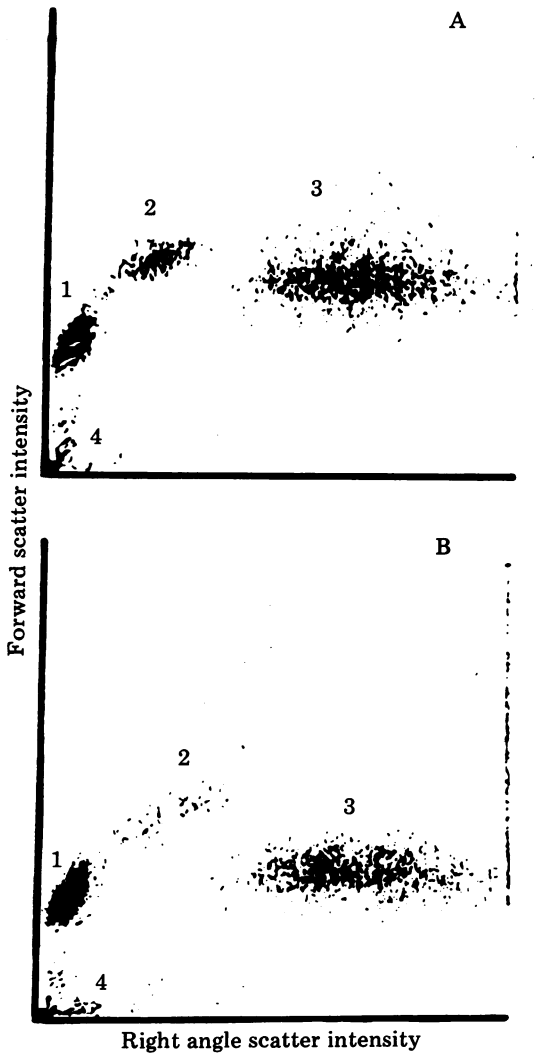

FIG. 2. Cytograms produced by the forward and the right-angle light scatter of a buffy coat preparation. Each dot represents a single cell. (*A*) Buffy coat preparation lysed with ammonium chloride lysing buffer. Clusters: 1, lymphocytes; 2, monocytes; 3, granulocytes; 4, aggregates of platelets and erythrocyte ghosts. (*B*) Same as *A* but after immunofluorescent staining.

This light scatter analysis can also be applied to whole blood.

Comparison of Data with Buffy Coat and E^+ Preparations. The fluorescence profiles of each monoclonal antibody reacting with the cells in the lymphocyte cluster were similar to those

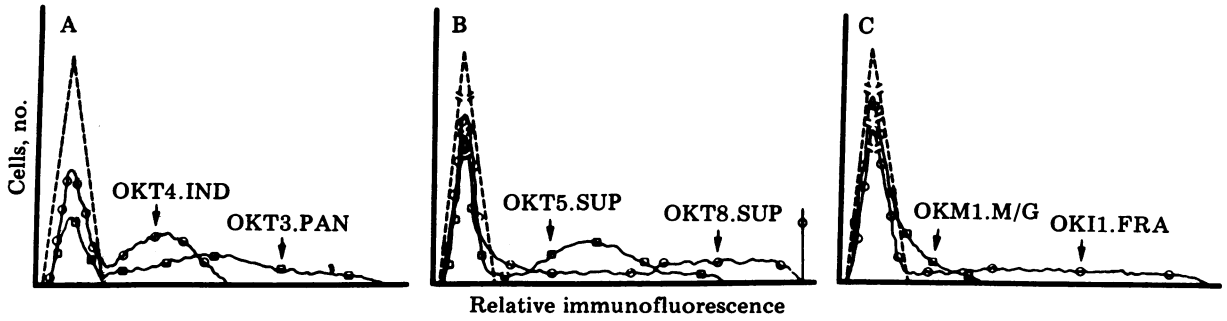

FIG. 3. Histograms of buffy coat cells treated with monoclonal antibodies. The broken line represents a control preparation incubated with ascites induced by the parent myeloma used for fusions.

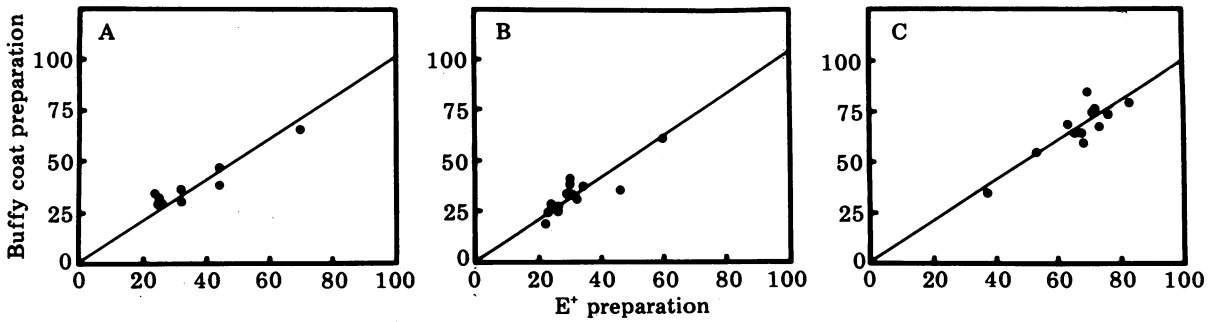

FIG. 4. Linear regression analysis comparing T-cell subclasses prepared by buffy coat and E^+ methods. (*A*) OKT8.SUP$^+$. *r* (Pearson's correlation coefficient), 0.95; *P* (probability that *X* and *Y* are uncorrelated), 0.0001; *m* (slope of the regression line, $Y = mX$, through the origin), 1.05; *n* (sample size in the analysis), 9. (*B*) OKT5.SUP$^+$. *r*, 0.86; *P*, 0.0002; *m*, 1.03; *n*, 13. (*C*) OKT4.IND$^+$. *r*, 0.087; *P*, 0.0001; *m*, 0.99; *n*, 13.

obtained with fractionated mononuclear or E^+ populations prepared from peripheral blood (Fig. 3).

Results obtained with the buffy coat and the E^+ preparations showed high correlations: $r = 0.87$ for OKT4.IND$^+$, 0.86 for OKT5.SUP$^+$, and 0.95 for OKT8.SUP$^+$ cells. The probabilities of unrelatedness between the two methods were all less than 0.001 (Fig. 4).

Analysis of T Lymphocyte Subclasses. Table 1 summarizes the results with buffy coat preparations on T lymphocyte subclasses of normal donors 20–65 years old. T cells, identified by OKT3.PAN monoclonal antibody, represented approximately 75% of cells in the lymphocyte cluster. The remaining 25% consisted of monocytes (OKM1.M/G$^+$, OKT3.PAN$^-$) and B lymphocytes (OKI1.FRA$^+$, OKT3.PAN$^-$, OKM1.M/G$^-$). OKT3.PAN identified all peripheral T lymphocytes, which comprise two separate subclasses represented by OKT4.IND$^+$, OKT5.SUP$^+$, and OKT8.SUP$^+$ cells. The percentages of these cells in the lymphocyte cluster therefore were normalized against OKT3.PAN$^+$ lymphocytes to obtain a percentage of the number of total T cells within each of the two subclasses. Thus, OKT4.IND, OKT5.SUP, and OKT8.SUP define 62%, 36%, and 43% of peripheral T lymphocytes, respectively. By comparison the E^+ preparation contained 88% T lymphocytes, 13% monocytes, and a few B lymphocytes.

DISCUSSION

Approximately 75% of the cells in the lymphocyte cluster (Fig. 2) are T cells (OKT3.PAN$^+$). The remaining 25% consist of B cells (OKI1.FRA$^+$), monocytes (OKM1.M/G$^+$ and some OKI1.FRA$^+$), and some null cells which lack all these markers (Table 1); the monocytes have been described (15) and, in fact, some of these appear to rosette with sheep erythrocytes to form 13% of E^+ populations. The OKT3.PAN$^+$ lymphocytes consist of approximately 60% inducer T cells (OKT4.IND$^+$) and 40% suppressor T cells (OKT8.SUP$^+$); the latter contain the smaller OKT5.SUP$^+$ population. The percentages of each T cell subclass obtained with buffy coat preparations are in good agreement with those obtained with E^+ preparations (Fig. 4).

The conventional technique of enumerating T-lymphocyte subclasses requires a working volume of at least 20 ml and may take as long as 2 days to obtain results. The method described in this report uses as little as 50 μl of whole blood for each antibody tested, and produces results on the flow cytometer within 2 hr. This improvement is attributed to optical and electronic assignment of peripheral leukocytes into discrete clusters (Fig. 2), the high-speed enumeration of immunofluorescent T cells on the flow cytometer, and the development of monoclonal antibodies to human T cells. Replacement of indirect immunofluorescent staining with direct staining could provide a further significant saving in time and effort.

The ratio between inducer T cells and suppressor T cells, which are reciprocal populations among the T lymphocytes, appears to be relatively constant in healthy volunteers. Preliminary studies suggest that this inducer/suppressor ratio may be an important measure of immunoregulatory status in man.

It is now clear that morphologically similar lymphocytes, circulating in the peripheral blood, contain functionally different T lymphocytes which can be further divided into inducer

Table 1. T lymphocytes, T lymphocyte subclasses, and other cell populations in buffy coat and E^+ preparations

Monoclonal antibody	No. samples tested	% of lymphocytes		% of OKT3.PAN$^+$ cells	
		Mean ± SD	Range	Mean ± SD	Range
		Fluorescent cells in buffy coat			
OKT3.PAN	40	76 ± 6	64–85	—	—
OKT4.IND	40	46 ± 8	33–55	62 ± 10	42–77
OKT5.SUP	40	27 ± 6	14–38	36 ± 8	18–50
OKT8.SUP	40	32 ± 8	24–37	43 ± 10	34–49
OKM1.M/G	40	14 ± 6	6–30	—	—
OKI1.FRA	40	11 ± 4	5–22	—	—
		Fluorescent cells in E^+ preparation			
OKT3.PAN	17	88 ± 7	65–96	—	—
OKT4.IND	17	57 ± 11	30–70	64 ± 12	37–83
OKT5.SUP	17	31 ± 5	15–49	33 ± 9	22–60
OKT8.SUP	13	31 ± 11	17–57	36 ± 13	25–70
OKM1.M/G	7	13 ± 4	4–20	—	—
OKI1.FRA	10	6 ± 3	1–10	—	—

(helper) T cells, cytotoxic T cells, and suppressor T cells (9–14). The distribution of these T lymphocyte subclasses, and hence the immunoregulatory status of the individual, is known to vary (16). Full evaluation of subclasses and their alterations with age and in different disease states require further investigation. The method described here offers a rapid technique for evaluating T cells and their subclasses, using a very small volume of blood, with the potential for longitudinal studies.

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