Cytometry 7:178–183 (1986)

Physical Discrimination Between Human T-Lymphocyte Subpopulations by Means of Light Scattering, Revealing Two Populations of T8-Positive Cells

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Received for publication June 25, 1985; accepted September 18, 1985

Light-scattering properties of human T-lymphocyte subpopulations selected by immunofluorescence were studied. Based on differences in orthogonal light scattering, two subpopulations of T8-positive cells can be distinguished. The first population (T8a) has the same orthogonal light-scattering properties as T4-positive cells, whereas the orthogonal light scattering of the second population (T8b) was about 70% larger. Orthogonal light scattering of Leu7-positive lymphocytes resembles that of the T8b population. We have studied the occurrence of the subpopulation in healthy individuals and we discuss their possible functional identification. Light-scattering properties of lymphocyte subpopulations in two patients with B-cell chronic lymphatic leukemia suggest that this observation is of clinical interest.

Key terms: T-lymphocyte subpopulations, light scattering, flow cytometry

Light scattering can be used to discriminate between different cell types. Forward light scattering is a measure for the size of the cell (5,13,23), whereas orthogonal light scattering is determined by internal and external cell structure (3,5,22,23). This is used in flow cytometry (FCM) of human leukocytes, where light scattering is mostly used to obtain a gate signal for lymphocytes, monocytes, or granulocytes (5,6,17,22). Small differences in light-scattering properties of B- and T-lymphocytes are observed by combination of UV and 488 nm forward light scattering (16). Changes in light scattering occur during human neutrophil activation (12). At present, determination of lymphocyte subpopulation distribution in human blood with monoclonal antibodies is used in a variety of studies (2,7,9,15,17,19,20). Until now, no differences have been reported in scatter signals of different T-cell subsets identified by monoclonal antibodies. Yet this may yield important information, since not all cells that react with a monoclonal antibody necessarily have the same morphological features. Recently, in fact, morphological differences between T4- and T8-positive lymphocytes have been reported (20). In this paper, we have therefore studied light scatter properties of lymphocyte subpopulations.

MATERIALS AND METHODS

Preparation of Lymphocytes

Human blood was collected by venipuncture into vacutainer tubes. This was done between 8 and 9 am in order to diminish the interday variation of T-cell subsets (4,8,19). For the anticoagulant, heparin (150 USP U sodium heparine/10 ml Venoject Terumo Europe NV) was used. A sample of 15 ml peripheral blood was diluted to 30 ml with minimal essential medium (MEM Flow Laboratories Ltd., Irvine, Scotland). With a syringe, 15 ml of lymphocyte separation medium (density 1.077 g/cm^3, Percoll Pharmacia Fine Chemicals Uppsala, Sweden) was carefully layered under the cell suspension and centrifuged at 20°C for 20 min at 1,300g. The lymphocytes in the interface were harvested, and residual erythrocytes were lysed for 20 min at room temperature (lysing buffer 8.29 g/l NH_4Cl, 0.0037 g/l Na_2EDTA, 1.00 g/l $KHCO_3$). Lymphocytes were washed three times in phosphate-buffered saline (PBS) and resuspended in standard buffer (PBS containing 0.005% sodium azide and 1% bovine serum albumin (BSA)). The concentration was adjusted to 1.10^7 lymphocytes/ml.

Immunofluorescence

The following purified monoclonal antibodies were used: T3 (T17-24-11, from National Institute for Public Health and Milieu (RIVM), Rijswijk, The Netherlands, anti-leu 4, from Becton Dickinson, Amersfoort, The Netherlands) pan T-cell marker, T4 (RIV 6, from RIVM)

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T helper-inducer marker; T8 (RIV 1, from RIVM, anti-leu 2a, from Becton Dickinson), T suppressor-cytotoxic marker. In addition, anti-leu 7 (from Becton Dickinson) biotin conjugate, which identifies a subpopulation of natural killer, killer, and large granular lymphocytes (1,11), was used. For second-step reagents, fluorescein-conjugated goat antimouse immunoglobulin (FITC GAM) (Central Laboratory of Blood Transfusion Service (CLB) Amsterdam, The Netherlands) and phycoerythrin- (PE) conjugated avidin (Becton Dickinson) were used. All reagents were pretitered by using normal lymphocytes to determine optimal conditions for binding.

Briefly, a 100-μl cell suspension was incubated with 20 μl diluted monoclonal antibody (antibodies were diluted in standard buffer) for 30 min at room temperature. The cells were washed twice with standard buffer and resuspended in 100 μl diluted FITC GAM. After incubation for 30 min on melting ice, the cells were washed twice and resuspended in 1 ml standard buffer and kept on ice until analyzed. In two-color fluorescence preparations, cells were resuspended in 100 μl standard buffer and incubated with 20 μl diluted anti-leu 7 for 30 min on ice.

Subsequently, cells were washed twice and resuspended in 100 μl diluted PE-conjugated avidin. After incubation on ice for 30 min, cells were washed twice and resuspended in 1 ml standard buffer.

Flow-cytometric analysis was performed the same day to preserve optimal binding of monoclonal antibody and light scatter properties of lymphocytes (14).

Flow Cytometer (FCM)

The FCM was built to our own design. It contained a flow cell with a 250 $\times$ 250 μm square flow channel (Precision Cells, Hicksville, NY). As a light source, we used a 3-W argon ion laser (model CR3, Coherent Radiation, Palo Alto, CA) tuned at 488 nm with a light intensity of 100 mW. The forward light scatter is detected with a photodiode (model Pin 10-D, United Detector Technology) between 2 and 17°. Orthogonal light scattering and fluorescence were collected with a Leitz microscope objective (H32, NA 0.6) on photomultipliers (Hamamatsu R928) provided with light diffusers. With this system, the maximum and minimum collecting angles were 115 and 65°, respectively. FITC emission was measured with a six-cavity bandfilter (510–550 nm, Pomfret Research Optics, Inc.). The PE fluorescence was measured with an OG 590 colored glass filter (Schott, Tiel, The Netherlands). For two-color fluorescence measurements, a 550-nm dichroic mirror (03 SWP017, Melles Griot, Irvine, CA, places at an angle of 45°) was used to separate the FITC from the PE emission.

The signals were analyzed with a homemade multichannel analyzer connected to a LSI 11/23 minicomputer (Digital Equipment Corporation). Data are stored as two-parameter histograms with a resolution of 80 $\times$ 80 channels. Data are represented as histograms or two-parameter density maps. Pixels with increasing size correspond to increasing occupancy of the channels. Five levels were chosen corresponding to 10, 30, 50, 70, and 90% of the maximum occupancy for Figure 1b,c and 0.014, 0.1, 0.7, 5, and 17% for Figure 3a. At least 20,000 cells are displayed in the figures.

FCM Measurement Conditions

In order to obtain reproducible conditions for the measurement of light-scattering histograms of lymphocytes on any given day, we searched for standard particles that had to conform to the following requirements: 1) small variation coefficient in forward scattering, right angle scattering and fluorescence signal; 2) easily excluded during lymphocyte measurements; 3) no influence on monoclonal antibody binding or light-scattering properties of lymphocytes; 4) stable for at least 1 yr.

Satisfactory results were obtained with 1.6 μm yellow green fluorescent monodisperse carboxylated microspheres (Polysciences Inc., Warrington, PA). The FCM was adjusted with these microspheres. The peak positions of forward scattering, right angle scattering, and fluorescence signals were placed on fixed channel numbers with optimal variation coefficients. Shortly before each measurement, we added 10^6 microspheres (10 μl of a solution of 10^8 spheres/ml PBS) to the sample.

During measurements, microspheres were exluded through discrimination on forward light scattering. Stability of the FCM during measurements was monitored on an oscilloscope with the microsphere signals.

The cut-off level for determinating the percentage of positive cells was chosen at such a level that less than 1% of the cells were positive in a control sample stained with FITC GAM.

Light scatter histograms were measured of lymphocyte subpopulations after gating on the fluorescence intensity of immunofluorescently labeled cells. Gates are chosen such that gated cells are definitely positive: less than 0.1% of the cells of control samples fall within the gates.

RESULTS

By using fluorescent beads as an internal standard, we were able to obtain reproducible histograms for forward and orthogonal light scattering of human lymphocytes. The day-to-day variation of the peak position of these histograms for a single healthy donor was less than 6% measured over a period of 1 month. When all lymphocytes were measured, the interdonor variation of the peak positions was about 6% (SD) for the forward and 9% (SD) for the orthogonal light scattering, whereas the shape of the histograms was very similar for all donors.

We have observed that minor changes in the preparation procedure can result in great variations in light scatter histograms. For instance, the use of MEM instead of PBS as a cell medium significantly increases the forward light scattering (about 50%). The same is true when buffy coat (anticoagulated with citrate) instead of venous blood (anticoagulant heparin) is used.

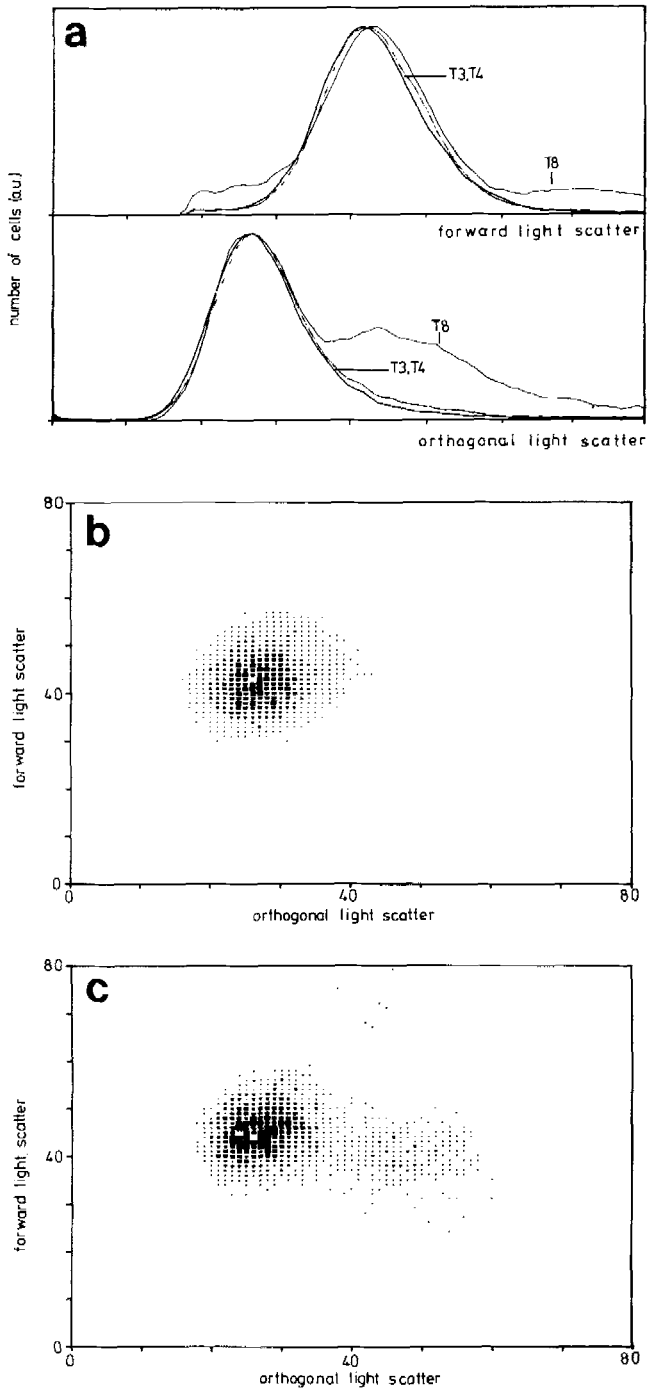

FIG. 1. a. Forward and right angle scatter histograms of T3-, T4-, and T8-positive lymphocytes of one healthy individual. Note the correspondence of peak-position and shape of the $T3^+$ and $T4^+$ histograms, whereas the $T8^+$ histogram reveals a second population in orthogonal light scatter. b,c. Density maps of forward and right angle scattering of, respectively, $T4^+$ and $T8^+$.

Fixation of cells results in a decrease of both forward and right angle light scattering.

Pronounced differences in light scatter properties were observed when different T-cell subpopulations where analyzed. In Figure 1 forward and orthogonal light scattering histograms of T3-, T4-, and T8-positive ($T3^+$, $T4^+$, $T8^+$) cells and density maps of $T4^+$ and $T8^+$ cells of a single donor are plotted. From Figure 1, it is evident that $T8^+$ cells consist of at least two subpopulations with different light scatter properties. The main difference is observed with orthogonal light scattering, the histogram of which shows two separate maxima. This was observed for all donors tested (n = 15); the relative contribution of the two populations was donor dependent. The percentages of each population were estimated by analyzing the measured histogram into two components and counting the number of cells in each population, as indicated in Figure 2. The two subpopulations are designated as T8a and T8b (see Fig. 2). It should be noted that there is a minor third population with a relatively intense forward and orthogonal light scattering in the $T8^+$ cells (Fig. 1c). This could be due to monocytes that are aspecifically stained or an unknown subpopulation of $T8^+$ cells.

The results obtained were independent of the source of the T8 monoclonal antibody (RIV 1; anti-leu 2a).

In Table 1, the percentages of lymphocyte subpopulations are listed for 15 healthy donors. It shows a large variation in the occurrence of $T8^+$ subpopulations in normal individuals.

In order to get more insight into the nature of the T8a and T8b subpopulations, we used two-color immunofluorescence with FITC-labeled T8 and PE-labeled leu7. The density map of this measurement is given in Figure 3a. By gating on the different fluorescence signals, histograms of the orthogonal light scattering of $T8^+$ cells, $T8^+leu7^-$ cells, $T8^+leu7^+$ cells, and $T8^-leu7^+$ cells are measured and given in Figure 4, b,c,d, and e, respectively. From Figure 4, it is clear that the $leu7^+$ cells have a large orthogonal light scattering that strongly resembles the T8b population and is identical to the $T8^+leu7^+$ cells. Interesting results were obtained when the light-scattering properties of lymphocyte subpopulations of patients with hematological disorders were analyzed. This is illustrated in Figure 4, in which orthogonal light scatter histograms of $T8^+$ cells of two healthy individuals are compared with those of two patients suffering from B-cell chronic lymphatic leukemia (B-CLL). The histograms of both patients resemble the T8b population of healthy individuals, whereas the T8a population is negligible.

DISCUSSION

In the present study, we have shown that reliable and reproducible measurements of forward and orthogonal light scattering of human lymphocytes can be obtained with a flow cytometer. Using this approach, we have identified for the first time light-scattering differences between T-cell subpopulations in healthy individuals. $T8^+$ cells can be divided into two subpopulations on the basis of light scattering (T8a,T8b, Fig. 1a,c). Hardly any changes are seen in forward scattering, whereas there exists a substantial difference in orthogonal light scattering between the two populations. The $leu7^+$ cells

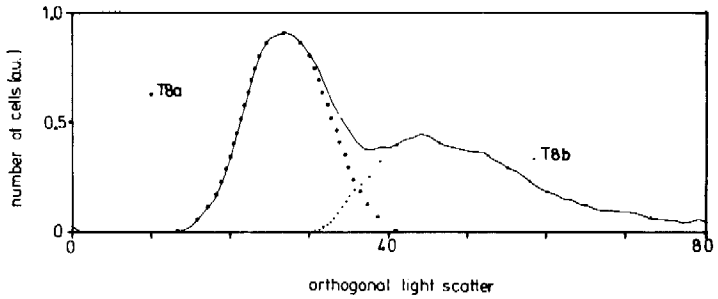

FIG. 2. Estimation of the percentage of T8 subpopulations. The histogram is divided into two components by making the component with low orthogonal scatter signal (T8a) symmetrical; subtraction of this component from the histogram gave the second component (T8b). The number of cells under each curve was counted.

Table 1
Comparison of T-Cell Subsets in 12 Healthy Donors and One Donor for 4 wk in Succession

	Percentage of total lymphocytes					Ratios		
Donor	T3 pan T	T4 helper cellset	T8 suppressor/ cytotoxic cellset	T8a[a]	T8b[b]	T4/T8	T4/T8a	T4/T8b
1a	74	48	23	ND	ND	2.1	ND	ND
1b	65	42	19	12	7	2.3	3.6	6
1c	72	51	20	12	7	2.6	4.2	7
1d	65	44	14	9	6	3.0	5.0	7
2	ND	60	23	13	10	2.6	4.7	6
3	ND	42	19	7	13	2.2	6.2	3
4	59	43	15	7	8	2.9	6.2	5
5	75	47	23	14	9	2.0	3.3	5
6	66	45	16	11	5	2.9	4.0	9
7	72	38	18	15	3	2.1	2.4	13
8	64	47	14	9	4	3.5	5.1	12
9	69	48	16	10	7	3.0	5.1	7
10	86	64	14	9	6	4.5	7.5	11
11	69	51	20	14	6	2.6	3.6	9
12	68	54	21	14	7	2.6	3.9	8
Mean	70	48	18	11	7	2.7	4.6	7.7
(SD/mean) × 100 (%)	9	14	17	24	36	23	28	36

[a]T8 cellset, low 90° scatter signal.
[b]T8 cellset, high 90° scatter signal.

show an orthogonal light scattering similar to that of the T8b population.

The correlation between the light scatter differences we observe and cell morphology is as of now unknown. Light scattered in the orthogonal direction contains information regarding internal and external cellular morphology (3,5,22,23). Orthogonal light-scattering signal increases when nuclear shape becomes more irregular and nuclear size and intracellular particles, e.g., granulation, increase (3). Leu7$^+$ cells and T8b$^+$ cells show an orthogonal light scattering that is on the average about 70% larger than that of the majority of lymphocytes (Fig. 3). This can be caused by cytoplasmatic granulation, which is a common feature of leu7$^+$ cells, and by irregular nuclear size and shape. The latter is supported by recent work of Tanke et al. (20), who reported that the T8$^+$ and leu7$^+$ fraction was strongly enriched for cells with irregular-shaped nuclei.

The rather large differences in light scatter properties of lymphocyte subpopulations reported in the present study have an important consequence for flow cytometric measurements of lymphocytes. When forward and orthogonal light scattering are used to select lymphocytes in a leukocyte preparation, the selected window setting must be a wide one, otherwise, certain subpopulations (e.g., leu7$^+$, T8b$^+$ cells) are missed.

Remarkable light-scattering properties of T-lymphocytes were observed for two B-CLL patients. In both B-CLL patients studied, the orthogonal light scattering of T8$^+$ lymphocytes was abnormal (Fig. 4). Peak positions of the orthogonal light-scattering histograms were within the normal range of the T8b population of healthy individuals. Functional and morphological studies of the two T8 populations are needed to determine whether or not the T8b population of healthy individuals has the same morphological and functional properties as that of

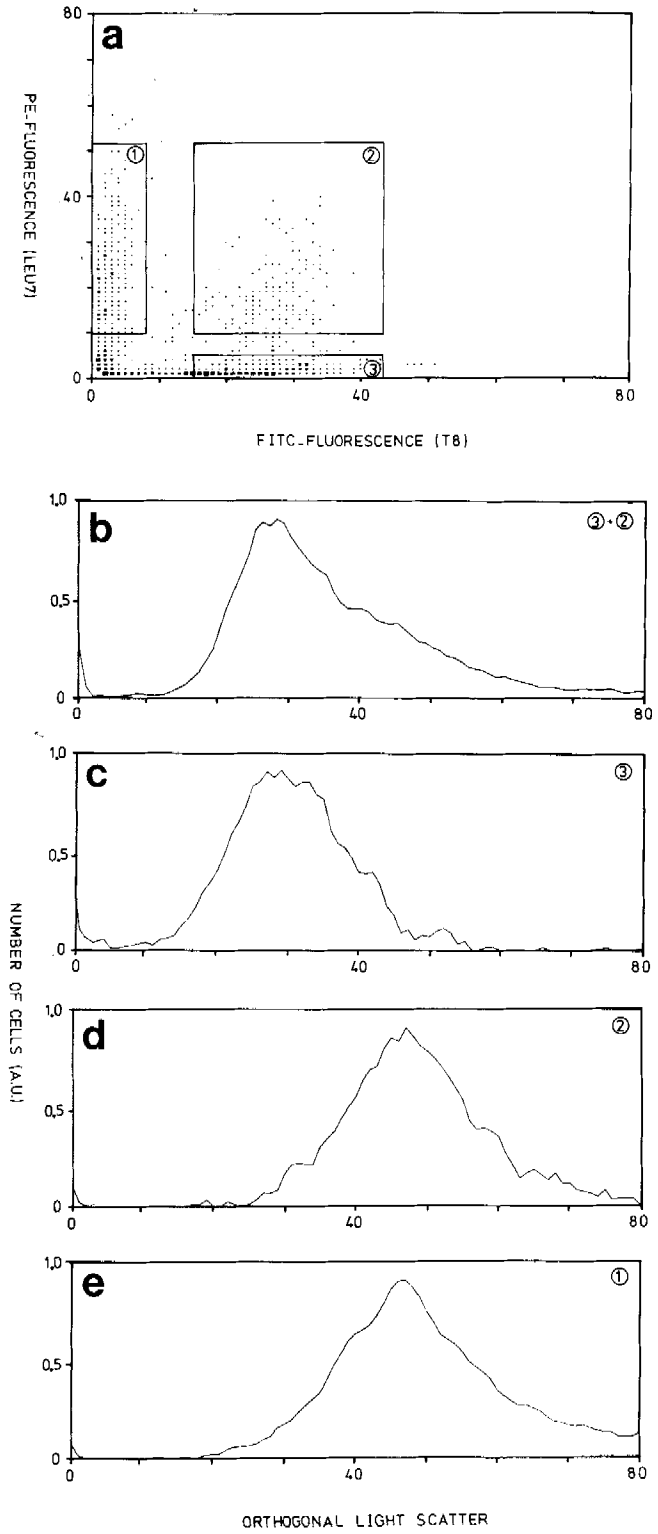

FIG. 3. a. Two-color immunofluorescence density map of FITC-labeled T8 and phycoerythrine-labeled anti-leu 7 of a healthy individual. Gates on $T8^+(2+3)$, $T8^+leu7^-(3)$, $T8^+leu7^+(2)$, $T8^-leu7^+(1)$ populations are indicated. b,c,d,e. Right angle light scatter histograms of the gated subpopulations, respectively, $T8^+$, $T8^+leu7^-$, $T8^+leu7^+$, $T8^-leu7^+$.

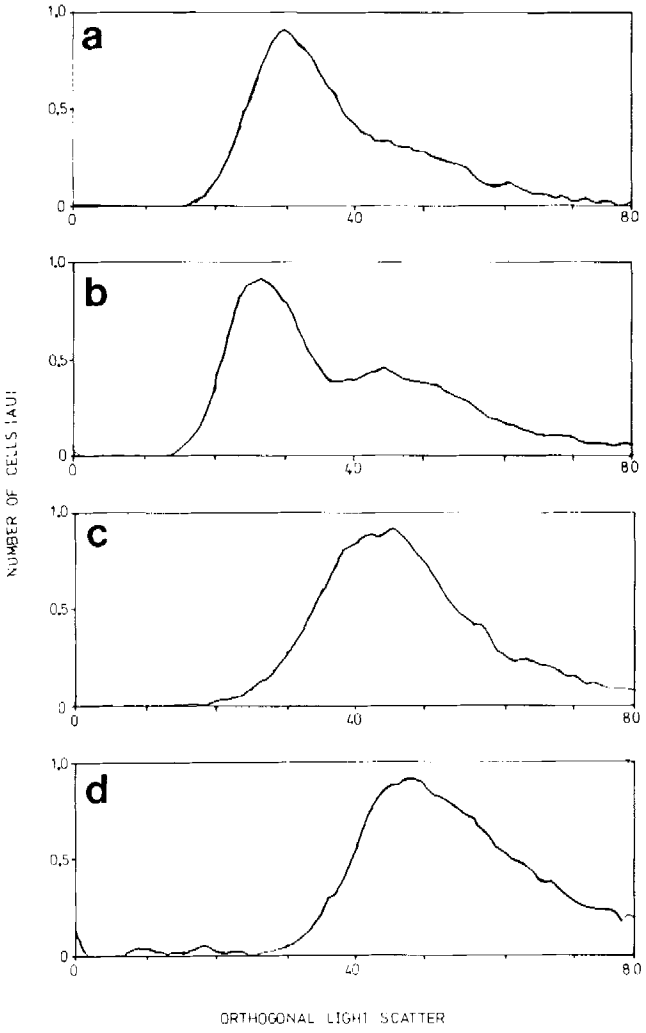

FIG. 4. Orthogonal light scatter histograms of $T8^+$ lymphocytes of two healthy donors (a,b) and two patients with B-CLL (c,d).

the B-CLL patients.

The functional identification of the two T8 subpopulations is of great importance. Since cells reacting positively with T8 form a heterogenous population and consist of suppressor cells and cytotoxic cells (10), a possibility is to identify the T8a population with $T8^+$ suppressor cells and the T8b population with $T8^+$ cytotoxic cells. The results obtained with the dual immunofluorescence measurements in Figure 3 support this hypothesis: At least those $T8^+$ cytotoxic cells identified by leu7 are of the T8b population. The majority of $T8^+leu7^-$ cells has the same orthogonal light scattering as the T8a population, whereas a minor fraction can be attributed to the T8b cells. In accord with this observation is the fact that in healthy donors the major fraction of $T8^+leu7^-$ cells consists of suppressor cells and a minor fraction consists of cytotoxic cells.

A more direct identification of the two T8 subpopulations will await further studies using more selective

antibodies and functional tests of cells selected on the basis of immunofluorescence and light scattering with a cell sorter.

The question of the identity of the two $T8^+$ subpopulations is important in view of the frequent use of T4/T8 ratios in many medical studies reported in the current literature (2,7,9,15,18,21). Although it is known that the T8 cells consist of two functionally distinguishable populations, the T4/T8 ratio is often taken as a measure of helper/suppressor activity. Since the $T8^+$ lymphocytes consist of a T8a and T8b subpopulation, at least three T4/T8 ratios can be determined: T4/T8, average value 2.7; T4/T8a, average 4.6; T4/T8b, average 7.7. If the T8a population consists of suppressor cells and the T8b population of cytotoxic cells, the T4/T8a ratio is the real helper/suppressor ratio. This ratio can be quite different from the T4/T8 ratio usually determined. For instance, in the case of the CLL patients, abnormal low values of T4/T8 ratios were found (0.6 and 0.9, normal 2–3,4). However the T4/8a ratios are strongly increased (61 and 24, normal 2,4–7.5) which, if the hypothesis stated above is correct, means that the helper/suppressor ratios are increased instead of decreased.

Light-scattering properties of lymphocyte subpopulations can easily be obtained, and they give valuable additional information on lymphocyte subpopulation distribution and identification. In the patients studied, strongly abnormal results were found. This suggests that determination of light-scattering properties of lymphocyte subpopulations is of clinical interest.

ACKNOWLEDGMENTS

The authors wish to thank all the volunteers for being so kind to spare their blood.

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