Three-Color Immunofluorescence Analysis of Leu Antigens on Human Peripheral Blood Using Two Lasers on a Fluorescence-Activated Cell Sorter

Michael R. Loken and Lewis L. Lanier
Becton Dickinson Monoclonal Center, Inc., Mountain View, California 94043
Received for publication June 24, 1983; accepted August 23, 1983

A fluorescence-activated cell sorter was modified to quantify simultaneously three immunofluorescence stains on a population of cells. A dye laser containing rhodamine 6G was used to obtain 600 nm light to excite Texas Red coupled avidin. An argon ion laser operating at 488 nm excited both fluorescein and phycoerythrin directly conjugated antibodies. The emission from these fluorophores could be independently quantified as demonstrated by the histograms generated by samples labeled separately with each of the three stains. This three-color detection system was used to analyze human peripheral blood mononuclear cells for the expression of three antigens: Leu 2, Leu 7, and Leu 11. Upon reanalysis of the list mode data, several discrete subpopulations of lymphocytes could be identified based on the quantitative expression of these three antigens. Of particular interest in the normal sample studied was a population of dimly labeled Leu 2+ cells which were predominantly Leu II+, a phenotype which is seen infrequently in normal individuals. This technique expands the combinations of antigens that can be studied at any one time and will facilitate the detection and functional analysis of cells in heterogeneous populations.

Key terms: Fluorescence-activated cell sorter; immunofluorescence; phycoerythrin; lymphocyte subpopulations; Leu 2, 7, 11

The combination of immunofluorescence and flow cytometry has become an integral tool in the study of biological systems, especially in the discrimination between cells in heterogeneous populations. This identification of unique cell and cell populations among a mixture of many different cells has been used to study functional subpopulations of lymphocytes involved in the immune response (12,20,21), to distinguish between different lineages of leukocytes comprising the hematopoietic system (5,16), to identify and classify leukemias and lymphomas (11), to distinguish tumor from normal cells (2,3), and even to identify fetal cells in maternal circulation (9). As the complexity of the heterogeneous systems increases, the requirement for better discrimination between different cells also increases. One method of enhancing the discrimination is to utilize individual monoclonal antibodies which each uniquely identify a particular subset of cells. There is, however, no assurance that such unique antibodies can be made, since a direct correlation between cell function and cell surface antigen expression may not always exist. This limitation may be diminished using multiple antibodies on a heterogeneous population, with the goal of identifying unique cell populations based on the correlated expression of multiple cell surface markers. This requires both the availability of fluorophores with distinct emission spectra which can be conjugated to antibodies and an instrument to identify independently two or more fluorophores on a cell population.

Several different techniques have been used to detect multiple staining of cells in flow systems. Two-color immunofluorescence can be quantified using a single laser system with fluorescein (FITC) and rhodamine as the fluorophores (17); however, the stains used in these studies must be relatively bright. Sensitivity is lost in this system since rhodamine is not excited at its optimum and the optical filters required to eliminate the scattered light from entering the fluorescence detectors reduces the efficiency of FITC detection. The recent use of phycoerythrin (PE) as an immunofluorescent stain has dramatically improved the ability of detecting two colors of fluorescence using a single light source (18). The use of a dual laser system reduces the dependency on only optical filters to discriminate the two fluores-
cence signals. By focusing two lasers at different points of the flow stream, time, spatial separation, and spectral differences can be used independently to detect two fluorophores (26). Fluorescein and either of the two derivatives of rhodamine, X-RITC or Texas red (TX red), are the dye pairs that have been used for two-color immunofluorescence with dual laser systems, argon and krypton (23,27). These two red-emitting dyes are more efficiently excited by a krypton laser than is the parent compound. A dye laser can be placed in the krypton laser to improve the excitation of TX red in these systems (19). The detection of multiple stains has been achieved using three independent lasers as excitation sources to excite three fluorophores on a cell population (25).

In this article we describe a technique to quantify three different immunofluorescence labels on a single sample. The three dyes, FITC, PE, and TX red, are excited by two lasers (an argon and a dye laser). The feasibility of such a system was demonstrated by correlating the cell surface expression of three human antigens (Leu 2, Leu 7, and Leu 11) on normal peripheral blood lymphocytes. These antigens are on different proportions of normal peripheral blood lymphocytes and they identify different types of cytotoxic cells (1,4,13). Anti-Leu 2 identifies the cytotoxic/suppressor T cells (4). While anti-Leu 7 (1) and anti-Leu 11 (14) both react with natural killer (NK) cells, they stain different numbers of cells from a single individual (13). These reagents were chosen to define more precisely the subpopulations of lymphocytes existing in peripheral blood by correlating the expression of these three antigens.

MATERIALS AND METHODS

Reagents and Staining

The monoclonal antibodies used in this study, PE conjugated anti-Leu 2a, FITC coupled anti-Leu 11a, and biotin coupled anti-Leu 7, were prepared at the Becton Dickinson Monoclonal Center, Inc. (Mountain View, CA). Texas red dye (Molecular Probes, Junction City, OR) was coupled to avidin (Sigma, St. Louis, MO) by the method of Hayakawa et al (8).

Normal human peripheral blood mononuclear cells were obtained by Ficoll-Paque sedimentation (Pharmacia, Piscataway, NJ). These cells were washed in phosphate buffered saline three times and $5 \times 10^6$ were placed in tubes for staining. The FITC conjugated antibody and PE conjugated antibody were added to the tube and the cells were incubated for 20 min. The samples were washed and biotin conjugated antibody was then added. After 20 min incubation, the cells were washed and TX red avidin was added. After an additional 15 min the cells were washed twice and fixed as previously described (15). Cold phosphate buffered saline (0.1 M phosphate, pH 7.3) containing 0.1% sodium azide was used in all dilutions and washes. All procedures were carried out at 4°C. All antibodies were used at concentrations that yielded maximal fluorescence.

Description of Instrument

The cell sorter used in these experiments was a FACS 440 (Becton Dickinson FACS Division, Sunnyvale, CA) with a dual laser optical bench. The lasers used were a model 164-05 Argon ion laser and a model 350 dye laser (Spectra Physics, Mountain View, CA). The 488 nm line of the argon ion laser operating at 400 mw was used to excite FITC and PE. The dye laser using rhodamine 6G was tuned to 600 nm (325 mw) for the excitation of TX red. The two beams were focused to 20 μm spots approximately 250 μm apart so that the emissions generated at these two points were separated temporally, spatially, and spectrally.

The absorption spectra (collected on a model 110 double-beam spectrophotometer, Hitachi Scientific Instruments, Mountain View, CA) and emission spectra (obtained on a spectrofluorometer, Perkin-Elmer, Norwalk, CN, model MPF-2a) of the dye conjugates used in this study are shown in Figure 1. Anti-Leu 2a-PE, avidin-Tx Red, and goat anti mouse IgG-FITC were used to obtain these spectra. The concentration of dye conjugate was adjusted so that the optical density of the solution

[Fig. 1. Comparison of absorption (A) and emission spectra (B) of fluorescein (FITC), phycoerythrin (PE), and avidin Texas red (TX red). Both the absorption and the emission curves were normalized at the peaks. The rectangles in B approximate the transmission windows of the band-pass filters (BP) used to separate the emission from the three dyes.]
was less than 0.1. These spectra were normalized for presentation on the same scale. Although 488 nm is not optimal for the excitation of PE it is sufficient to excite this dye for two-color immunofluorescence experiments (18). The choice of 600 nm excitation on the dye laser was made because this was near the peak excitation of TX red, yet above the emission peak for PE. The approximate spectral characteristics of the optical filters (Becton Dickinson FACS Systems) used in this study are also shown in Figure 1. The arrangement of these filters on the optical bench of the cell sorter is shown in the schematic diagram (Fig. 2). Filter BP (band pass) 530 (#19-62774-07) was placed in holder #1 in the green channel along with BR (Band Reject) 514 Omega Optics (Brattleboro VT). Filter BP 575 (#19-62774-02) was used in the orange channel, holder #2, and filter BP 630 (#19-62774-09) was placed in the red channel, holder #3. A dichroic mirror DM 560 (#19-62772-00) was used to deflect the green light, mirror #1, while a split mirror, #2 (19-62781-00), was used to separate the orange and red emission. Logarithmic amplifiers were used to process the signals. A compensation network (17) was used to eliminate the orange emission of FITC from the orange, PE, channel. No PE emission could be detected in the green channel.

![Fig 2. Schematic drawing showing the filter arrangement in the fluorescence detection system. The approximate transmission characteristics for filters #1, 2, and 3 are illustrated in Figure 1. The dichroic mirror #1 is a long pass filter reflecting light below 560nm. Since the orange and red emitting dyes are excited by two different lasers focused at different points on the stream, the split mirror (#2) separates the orange and red emission based upon spatial differences in the two optical paths. The separation of the three colors is illustrated by the hatched, lightly and darkly stippled lines.](image)

The data were collected as a four-parameter list on a Consort 40 data management and control system (Becton Dickinson) with these assignments: parameter #1 = Forward angle light scattering; parameter #2 = green (FITC); parameter #3 = Red (TX Red); parameter #4 = orange (PE). For the single-stain experiments shown in Figure 3, data from 10,000 cells were collected. In the triple-staining experiment (Figs. 4–6) 100,000 cells were examined. Figures 4–6 were all obtained from the same list of data with different criteria set for the reprocessing of those data.

**RESULTS**

**Independence of Detection of Three Fluorophores**

As in any multiple-staining experiment, it was necessary to show that each stain could be detected in the presence of the others and that the emission from one stain would not be observed in the other two detectors. Four samples, one with no stain added and three with each single stain alone, were prepared and analyzed on the FACS. The reagents used in these experiments were anti-Leu 11a FITC, anti-Leu 2a PE, and anti-Leu 7 TX red. A comparison of the histograms from these single-labeled samples can be made from Figure 3. The fluorescence histograms were gated on forward angle light scattering in order to identify the stains that were on lymphocytes and not on monocytes (22). The background autofluorescence of unstained cells is shown in Figure 3A. When only FITC labeled anti-Leu 11 was used to stain the cells, a compensation network was necessary to eliminate orange fluorescence emitted from this fluorophore from the PE channel (Fig. 3B). No compensation was needed for the TX red or PE single-labeled cells (Fig. 3C,D). These data demonstrate that each individual stain could be detected independently from the two other fluorochromes.

**Analysis of Triple Stained Cells**

A triple-labeled sample was then analyzed in order to correlate the binding of three reagents to the various cells in the sample. The fluorescence histograms of the triple-stained cells, gated again on forward angle light scattering, are presented in Figure 4. Comparing these curves with those in Figure 3 demonstrates that the profiles of each individual stain did not change when a single reagent or all three reagents were used to label the cells. (It should be noted that the data in Figure 3 was obtained from 10,000 cells while the data in Figure 4 came from 100,000 cells. This explains the difference in the appearance of the curves, which results from statistical counting errors). The identity in the corresponding histograms from the single- and triple-stained samples also indicated that the binding and emission of the three reagents was independent.

The correlations between the three different stains is shown in three scattergrams (dot plots) pairing two of the three stains in each one. The correlations between Leu 2 vs Leu 7 and Leu 2 vs Leu 11 are shown in Figure 5. From the Leu 2 vs Leu 7 dot plot (Fig. 5A) all four of
Fig 3. Comparison of histograms from single-labeled samples. Cells were analyzed after labeling with (A) no antibody, (B) FITC-conjugated anti-Leu 11 only, (C) biotin coupled anti-Leu 7 and TX red-labeled avidin only, and (D) PE-conjugated anti-Leu 2 only. The forward angle light scatter was collected as a linear parameter and was used to identify lymphocytes (gates shown in A). Logarithmic amplifiers were used to obtain the fluorescence histograms. The FITC and PE channels have 4 decades displayed full scale while the Texas Red channel has 2 decades in 256 channels. The cell numbers represented in the forward scatter histograms were half of the cell numbers in the fluorescence histograms. Each single-labeled sample was detected only in the appropriate fluorescence channel.

Fig 4. Histograms from triple-labeled cells. Mononuclear cells were labeled with FITC-conjugated anti-Leu 11, PE-conjugated anti-Leu 2, and biotin-conjugated anti-Leu 7 followed by TX red avidin. The lymphocyte gates in the light scatter channel used to generate the fluorescence histograms are shown in the upper left quadrant. The gates were used to denote Leu 2 negative, Leu 2 dull, and Leu 2 bright in the lower right quadrant. The scales on the abscissa for each histogram are the same as shown in Figure 3.
the following phenotypes were identified: Leu 2−7−, Leu 2+7−, Leu 2+7+, and Leu 2−7+. The correlation plot between Leu 2 and Leu 11 (Fig. 5B) indicates that the cells that expressed large amounts of Leu 2 did not have Leu 11. There were, however, dimmer Leu 2+ cells which expressed the Leu 11 antigen and also cells which bound anti-Leu 11 but did not have Leu 2 on their surfaces. Although the distinction between Leu 2 bright, dim, and negative is arbitrary (boundaries are illustrated in Fig. 4), there was a consistent difference in the binding of the anti-Leu 11 to these cells.

The primary purpose of this experiment was to correlate the cells that expressed the Leu 7 and Leu 11 anti-

gens (Fig. 6). When the list mode data were analyzed for all lymphocytes (Fig. 6A) four phenotypes of cells were identified by their Leu 7,11 expression: Leu 7−11−, Leu 7+11−, Leu 7+11+ and Leu 7−11+. The proportion of the cells in each of the quadrants of Figure 6 are presented in Table 1.

The relationship between these two stains and the third label, Leu 2, must be found using double-gating on lymphocytes and on Leu 2 expression (Fig. 4). The correlation between Leu 7 and Leu 11 for Leu 2 negative cells is shown in Figure 6B. Cells of the Leu 7+11+ and Leu 7−11+ phenotypes were clearly demonstrated, whereas there were fewer Leu 7+11− cells than in the entire lymphocyte gate (Table 1). This is in contrast to the dot plot obtained from the Leu 2 bright cells (Fig. 6C), where there were very few Leu 7+11+ and Leu 7−11+ cells. By gating on the dim Leu 2+ cells, the correlation between the Leu 7 vs Leu 11 expression were obtained (Fig. 6D). There were fewer Leu 7−,11− and an increase in the Leu 7+11+ and Leu 7−11+ populations.

The proportion of cells bearing the four cell surface phenotypes based on Leu 7,11 expression are presented in Table 1. The percentage of all lymphocytes that were within the Leu 2 gates are also presented so that the total representation of cells with each unique phenotype based on the three markers can be calculated. From these data it can be seen that the bright Leu 2+ cells did not express the Leu 11 antigen. In contrast, the dull Leu 2+ cells were predominantly Leu 11 positive. Interestingly the Leu 11+ cells were split evenly by the Leu 7 marker, both in the Leu 2− and the dull Leu 2+ populations. The Leu 7+11− were equally represented in the Leu 2 negative and the Leu 2 bright populations. The Leu 7+ cells which were Leu 2 dull almost all had the Leu 11 marker.

DISCUSSION

The identification of multiple stains on a single sample has been reported previously using either multiple lasers (24,25) or a mercury arc lamp (7). In these studies relatively bright cytochemical stains were used to identify multiple cellular parameters rather than the dimmer immunofluorescence stains. The data presented here illustrate that three-color immunofluorescence can be quantified in a flow cytometer using two lasers.

The crucial components in this system were the ability to excite both FITC and PE with a single argon laser and the use of a dye laser tuned to a wavelength above the emission of these two dyes. By selecting the appropriate optical filters for the PE channel, the 600-nm light from the dye laser could be excluded from this detector. This is a wavelength that is still efficient at exciting TX red. A similar three-color experiment was attempted using argon and krypton lasers; however, the 568 nm light from the krypton laser could not be eliminated from the PE channel without sacrificing sensitivity and resolution of the dimly stained cells.
Fig 6. Scattergrams correlating Leu 7 and Leu 11 expression. This correlation differs based upon the relative amounts of Leu 2 expression (see Fig. 4): A. All lymphocytes, B. Leu 2 negative, C. Leu 2 bright, D. Leu 2 dull. The proportion of cells in each of the boxes are presented in Table 1.

<table>
<thead>
<tr>
<th>Gated On$^b$</th>
<th>LEU 7-11-</th>
<th>LEU 7+11-</th>
<th>LEU 7+11+</th>
<th>LEU 7-11+</th>
</tr>
</thead>
<tbody>
<tr>
<td>All lymphocytes</td>
<td>79.3</td>
<td>4.3</td>
<td>7.5</td>
<td>9.3</td>
</tr>
<tr>
<td>Leu 2 negative (72.6$^c$)</td>
<td>83.1</td>
<td>2.8</td>
<td>6.3</td>
<td>8.3</td>
</tr>
<tr>
<td>Leu 2 bright (18.6)</td>
<td>88.0</td>
<td>10.3</td>
<td>.8</td>
<td>.8</td>
</tr>
<tr>
<td>Leu 2 dull (8.9)</td>
<td>37.5</td>
<td>4.3</td>
<td>27.9</td>
<td>31.0</td>
</tr>
</tbody>
</table>

$^a$The cell surface phenotypes are defined by the correlation plots shown in Figure 6. The numbers given are the percentage of cells within the boundaries shown in Figure 6 based on the total number of cells that satisfy the gating criteria.

$^b$The gating parameters are illustrated in Figure 4. The correlation plots Leu 7 vs Leu 11 based on these gates are shown in Figure 6A-D.

$^c$The relative number of all lymphocytes that lie within the Leu 2 gates are shown in Figure 4.

The independence of the detection of each stain was demonstrated by the ability to detect single-labeled cells in only the appropriate channel. In addition, the histograms of the single-labeled cells were identical to the corresponding histograms in the triple-stained sample. This indicated that there was no interaction between the fluorophores when the three stains were used in combination. A compensation network was required only to remove fluorescein emission from the PE channel since there was significant orange emission from this fluorophore. By gating on the appropriate time delay the spectral overlap between PE and TX red did not affect the histograms, since these two dyes were excited by two different lasers at different times.
There are other dyes that could be used to replace TX red in a three-color immunofluorescence detection system. R Phycocyanin, excited at 600 nm with an emission at 640 nm, may be more efficient than TX red (18). Allo phycocyanin with emission at 680 nm can also be used as the third dye, but optimal excitation of this dye is at a longer wavelength than the 600 nm light used for these experiments (18). The combination of FITC, PE, TX red (or R phycocyanin), and allo phycocyanin could be used for four-color immunofluorescence. The first two dyes could be excited by the argon ion laser while the second two dyes could be excited by a dye laser. The overlap of spectra of R phycocyanin and allo phycocyanin would necessitate a second compensation network for this red-dye pair.

The application of the three-color immunofluorescence technique to the correlation of Leu 2, Leu 7, and Leu 11 revealed populations that could not be detected by any other technique. The cells from the individual used in this study were exceptional in two ways as compared to most normal donors. First, the individual used in this study had a significant population of Leu 2 dull cells which expressed the Leu 11 marker. (Fig. 5B). In most normal individuals studied, this population was not detected either because the dim Leu 2 positive cells were indistinguishable from the negative cells or because the number of cells in this population was too small to be identified (13). Although this dull Leu 2+ population was not discernable in the single parameter histogram (Fig. 4), the population was evident in the two-color correlation plot in Figure 5B. This demonstrates the added information obtained in correlating two parameters. Second, the proportion of Leu 7 cells among normal individuals usually exceeds the number of Leu 11 cells (13). The person in this study had more Leu 11 than Leu 7 cells, which could be accounted for by the dull Leu 2+11+ population.

The existence of the dull Leu 2+ cells demonstrates the requirement to correlate the cellular parameters on a quantitative as well as a qualitative basis. It is not sufficient to categorize cells as positive or negative. The amount of antigen expressed on any particular cell may also be significant. This type of quantitative analysis has been important in describing different subpopulations of B lymphocytes in the mouse based on levels of IgM and IgD expression (6,10).

Although three two-color experiments could have been used in an indirect manner to correlate these three antigens, more information is obtained from the direct three-color staining. The absence of the Leu 7+11+ and Leu 7−11− among the bright Leu 2+ cells (Fig. 6C) could have been predicted from the two-color data presented in Figure 5B since there is a lack of bright Leu 2+,11+ cells. The Leu 7+,11− cells in both the Leu 2+ and the Leu 2− populations could not be inferred from the two-color data. The determination that the dull Leu 2 cells which expressed the Leu 7 marker were predominantly Leu 11+ (Fig. 6D) also could not be determined by the two-color staining experiments.

In this experiment the cells that expressed the Leu 11 antigen were split into two groups by the Leu 7 marker regardless of whether they expressed none or small amounts of the Leu 2 antigen. It is not known if these two markers define specific lineages or if they represent different maturational stages of the same lineage. The functional studies determining the cell surface phenotype of the NK cells suggest that the most active of the cells are Leu 7−,11+ (13). It is not known if the dull Leu 2+,7−,11+ fall into this category or if they are a different type of cell.

The ability to correlate three antigens simultaneously suggests the possibilities of studying these unique and infrequent phenotypes in a direct manner and of sorting these cells for functional studies. This technique will permit more precise determination of subpopulations of cells and, when correlated with functional data, will allow a more complete dissection of the cells responsible for the immune response.

LITERATURE CITED

15. Lanier LL, Warner NL: Paraformaldehyde fixation of hemat-


