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# Rapid Multivariate Analysis and Display of Cross-Reacting Antibodies on Human Leukocytes

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**We present an application which can rapidly determine the binding patterns of monoclonal antibodies on mixed populations of cells simultaneously in a single rapid analysis. It is an application of the tube identifier parameter (TIP) system which can provide fully correlated list-mode data of the entire patient phenotype in a single file. Using the *phenogram* analytical display, we are able to determine the cross-reacting antibodies for an entire antibody panel for each cell type. This information can be displayed in a single plot. Using light scatter gating to select different populations of lymphocytes, monocytes, and neutrophils, phenograms can be simultaneously generated. This provides a directly comparable means of displaying the positive and negative binding characteristics of each antibody on each cell population. Any marker combination that is abnormal will be identifiable in the phenogram. Additionally, by plotting the fluorescence distributions of each marker beside one another (termed overview), quantifiable differences in intensity can be determined. There are 3 major benefits of the proposed analysis. By using the TIP concept, several sets of antibodies can be compared simultaneously. Any light scatter gate can be used and this gate can be changed on one histogram or plot, yet apply to the total analysis. Data analysis is particularly rapid since the entire phenotype of a patient can be evaluated by performing a single rapid analysis.**

**Key terms: Flow cytometry, TIP, immunophenotyping, phenogram**

Application of the tube identifier parameter (TIP) provides the ability to rapidly scan multiple populations of cells for antibody binding and therefore determine binding patterns (16). The unique nature of TIP is that since all of the information from all tubes is combined during collection on the cytometer, a greater proportion of the analysis is immediately available upon completion of the run. Complex analyses that take hours using traditional flow cytometry techniques can now be performed in minutes giving the user the opportunity to perform evaluations and comparisons previously considered too time consuming. Most current conceptions of flow cytometry data analysis are based upon determinations of 2 or 3 specific subsets within a single tube. This is because we are accustomed to thinking of flow cytometry as a single tube/file/histogram concept. Furthermore, available software restricts multivariate analyses to the events in a single list-mode file, which is usually representative of a single tube from a multi-tube set. Obtaining the relationships present from this multi-tube set requires repetitive iterations of the analysis steps. Combining the selected data to view the desired analyses requires further post analysis manipulations.

A considerable effort has been made in recent years to standardize methods for flow cytometric phenotyping in clinical laboratories (2,5,7,11–13,15,17). Problems have been identified such as improper gating of a population of cells which may alter the percentage of fluorescence positive cells identified in a particular light scatter gated population. In such situations, it is customary to reanalyze several files from that patient because identification of the abnormalities may not be detected until the data analysis stage. A critical question in immunophenotyping concerns obtaining correct percentages of positively labelled cells. Invariably,

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light scatter gating is used for much of the phenotyping performed (9,10,14). All of the techniques presently available such as the use of the CD45/CD14 combination for gating (8), back-gating techniques, and recurring multiple scatter and fluorescence gating can be applied in this application in addition to the other capabilities described.

There are many situations where identification of cross-reacting antibodies on different populations of cells would provide valuable information. This paper describes the application of a recently developed technique to evaluation of multiple sets of monoclonal antibodies across different cell populations. Of critical importance is the ability to provide rapid information in a correlated format such as presented in the phenogram. This phenogram can display a summary of all the binding patterns of each monoclonal antibody from a set of 20 to 30 different antibodies in one plot which can be displayed as samples are run on the cytometer.

The applications presented in this paper use a particularly rapid method for evaluating cross-reactivity of all the antibodies used in a panel (i.e., all 10 or 12 tubes comprising 20–30 antibodies) with all cell types. Further, it is important to view particular combinations of antibodies together as a set for comparative analyses. Because of the unique techniques employed, all of this including printouts and statistical information can be achieved in a single analysis of one TIP file in just a few minutes.

## MATERIALS AND METHODS

### Definitions

Because of the number of new terms discussed in this paper the following serves to recapitulate this information. The TIP concept has been described previously (16). Briefly, the technique is an alternative method for running samples on the flow cytometer. As an example of its operation, consider a patient for which 15 tubes of different antibody combinations are to be run. Each tube is presented to the cytometer normally; however, in addition to scatter and fluorescence variables, an additional parameter, TIP, is collected for each cell in each tube. Thus, each of the 15 tubes will have a unique value for TIP (1–15) embedded and correlated with all cells from that tube. All of the tubes are run consecutively and saved as a single large composite list-mode file. Only one file containing all the data is saved for the entire set of tubes. Cells from any antibody combination can be identified using the TIP value. TIP can also be used for primary or secondary gating: This means that if one light scatter population such as lymphocytes is of interest, by placing a single scatter gate, any antibody from any tube in the set can be evaluated simultaneously. Combinations of scatter, TIP, fluorescence, or time gates can be used in any sequence, on any tube in the set. Thus, future use of the term TIP in this manuscript refers to this technique.

Other definitions pertinent to this technique are also described briefly, although they are also previously described (16). *Overview* is a display format whereby the entire distribution of a particular variable for multiple tubes is set out as one display. For instance, in the example described above, all of the FITC fluorescence distributions for the lymphocyte population for each FITC-conjugated antibody could be set out one under the other by displaying fluorescence vs. TIP. These displays appear as dot plots of intensity distributions which are in a patterned format whereby color or different hatching indicate cell number at that channel location. More dense patterns indicate increased cell numbers. The *phenogram* is an analytic presentation of correlated information based upon the PRISM parameter. *PRISM* is a hardware method for determining correlations between multiple variables (1,18). For 2 variables, PRISM collects the equivalent of quadstat information in real time. This means it determines the percentage of cells that are $--$, $-+$, $+-$, and $++$ for any 2 variables. For more than 2 variables, however, PRISM provides much more than quadstat information. For instance, using 3 color flow cytometry, quadstats performed on each of the 3 possible bivariate plots cannot provide trivariate correlated information. PRISM can rapidly perform these complex correlations. Thus, the phenogram is the display created by plotting the TIP parameter against the PRISM parameter. These calculations are made as samples are run on the cytometer, and therefore the phenogram is immediately available for all tubes in the set upon completion of sample running. The phenogram can be displayed on the cytometer screen for real time monitoring of the entire phenotypic profile.

Flow cytometry was performed using an EPICS Elite cytometer (Coulter Cytometry, Hialeah, FL). Fluorescence was collected using an air cooled 15 mW argon laser (Cyonics Model 2201, San Jose, CA) operating at 488 nm for excitation. A Watson/Arnold flow cell (Profile flow cell—Coulter Cytometry Hialeah, FL) was used for all measurements. Data analysis was performed on the Elite workstation (80386, 16 Mhz) using the standard Elite software for analysis of all TIP files.

### Antibodies

Antibody combinations used are listed in Table 1. They consisted of either Cytostat or Coulter Clones (Coulter Cytometry, Hialeah, FL). The CD16 was from GenTrak, Inc., and it was subsequently shown not to be CD16 but most probably CD58. The method used to stain leukocyte subsets was as follows: 100 μl of peripheral blood was placed into a 12 × 75 mm tube and 10 μl of Cytostat or 5 μl of Coulter Clone was placed into the tube. After vortexing for 1 s, an appropriate additional antibody was added to the tube, vortexed for 1 s, and incubated for 10 min. In some cases, combinations of dual labelled antibodies were used.

After the incubation period, whole blood lysis was carried out using the Coulter Q-prep instrument on the 90 s cycle which also fixes cells (3). Tubes were left at

Table 1
*Set of Antibody Combinations Run for This Study*[a]

| Tube | PE | FITC | |
|---|---|---|---|
| 1. | IgM | $IgG_1$ | Control |
| 2. | CD14 | CD45 | |
| 3. | $IgG_1$ | $IgG_1$ | Control |
| 4. | CD4 | CD8 | |
| 5. | CD45RA | CD4 | |
| 6. | CD29 | CD4 | |
| 7. | CD56 | CD8 | |
| 8. | CD2 | CD19 | |
| 9. | Leu11c | Leu11a | |
| 10. | CD13 | CD16[b] | |
| 11. | $IgG_1$ | $IgG_{2a}$ | Control |
| 12. | CD2 | CD20 | |
| 13. | CD3 | HLA-Dr | |
| 14. | $IgG_1$ | IgM | Control |
| 15. | CD13 | CD11b | |
| 16. | Calibration beads | | Control |

[a]Each antibody pair is preceded by a matched isotype control tube from which appropriate gating was accomplished. All tubes were run as a single set and saved to a single TIP file. The entire set could then be re-analyzed as a single entity rather than as a succession of separate files each representing an antibody combination. The markers match the order in Table 1. Tube 1 is always at the bottom and tube 15 is always at the top of each display showing TIP as the Y axis parameter. Tube 16 (calibration beads) is not displayed on any of the histograms or phenograms. CD45RA-Coulter 2H4; CD29-Coulter 4B4; CD13-Coulter MY7; HLA-DR-Coulter 13; CD11b-Coulter Mo1; Leu11a, Leu11b-Becton-Dickinson.

[b]This CD16 was purchased from GenTrak and was later determined not to be CD16 but probably CD58.

room temperature in the dark for a minimum of 30 min before being run on the cytometer.

### Flow Cytometry

The cytometer was calibrated using level III Immunobrite beads (Coulter Cytometry, Hialeah, FL) after setting appropriate scatter detector and fluorescence photomultiplier tube (PMT) voltages. Color compensation was set using the 2 color combination CD4-FITC/CD8-PE tube and single color labeled cells. Once the compensation percentages were set, they remained unchanged for all subsequent samples in that set. Filter combinations used were 525 nm band pass for FITC fluorescence and 575 nm band pass for PE fluorescence. Signals collected included forward light scatter (FALS), 90° light scatter (90°LS), log green fluorescence (FITC), log red fluorescence (PE), TIP, TIME, and PRISM. In all, 7 fully correlated variables were collected for every cell (i.e., ungated list mode). A minimum of 5,000 lymphocytes were collected for each tube; however, data were collected for all cell types present.

## RESULTS

For each blood sample obtained, a complete panel of 15 tubes containing different monoclonal antibody combinations was run including control tubes as described in Table 1. Figure 1 shows the most rapid and complete analysis obtained in a single step analysis. This figure shows a screen shot of displays present on the cytometer immediately after the last tube of the 15 tube set was run. Any histogram or plot can be displayed by creating a profile suited to the particular analysis. This profile shows 6 plots, three of which are capable of representing the entire patient phenotype for each of lymphocytes, monocytes, and neutrophils. Additionally, the scatter and gates used to create the gated phenograms are shown. The three phenograms in the top row of Figure 1 represent lymphocytes (X scatter gate), monocytes (W scatter gate), and neutrophils (V scatter gate). Four vertical columns separate the different phenotypes. Each "spot" on the phenogram represents the cells positive for a particular phenotype, being cells $--$, $-+$, $+-$, and $++$ for FITC and PE fluorescence, respectively. The spots are displayed as contours where the number of contour lines represents the percentage of cells—more contour lines mean more cells in that "spot." If a position is blank, this indicates that no cells (or less than 2%) fall within this possible marker-cell combination. Several types of patterns are possible including dot densities, contours, and color codes. Each row in the phenogram represents a different tube in the set starting from tube 1 at the bottom to tube 15 at the top of each phenogram.

The first phenogram is for lymphocytes. One of the unique features of the phenogram is the ability to create fully correlated displays from the phenogram itself. As an example we have shown cells gated from row 2 (CD45-CD14 antibody combination—tube 2). The spot on the phenogram representing cells that are $CD45^+$ but $CD14^-$ is a dense spot (the gated area T). However, there is no spot in the $CD45^+/CD14^+$ area (gated area H). Since this is the phenotype for monocytes, the area should be blank in the lymphocyte gated phenogram. A second unique feature of the phenogram is that gating regions can also show variables that are "negatively selected." To demonstrate this concept the last two displays of Figure 1 show the two phenotypes described above. However, it is obvious that cells that are $CD45^+$ and $CD14^+$ are not lymphocytes; yet by gating on the lymphocyte phenogram, but *NOT* applying a scatter gate, any cell that would qualify for the phenotype selected will be displayed, regardless of scatter characteristics. This is how monocytes can be displayed from the blank area in the lymphocyte phenogram. This provides a powerful combination for multivariate analysis. The third phenogram (top right of Fig. 1) shows the same information for neutrophils. The actual percentage of cells for each spot in the phenogram can be printed out directly from the saved phenogram if necessary. The phenograms of other normal humans showed similar patterns to those represented above indicating that the patterns themselves may be useful for determining abnormalities.

There are several ways that TIP provides a complete

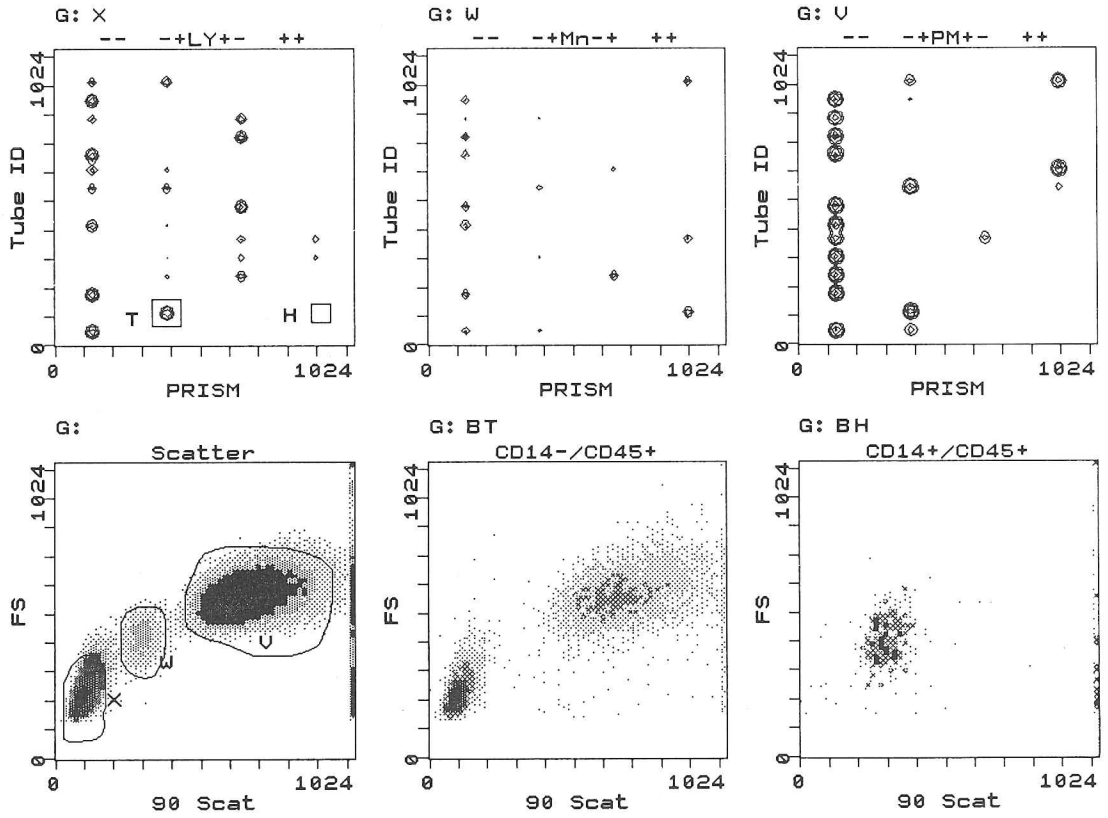


FIG. 1. Figure 1 is a screen shot of a single screen. No post-collection analysis has been performed on these data, they are shown as they appear on the cytometer screen immediately after sample collection. Three *phenograms* are displayed on the **top row.** Phenogram A is gated on region X and therefore represents all combinations of lymphocytes for each marker. The center phenotype is gated on monocytes (area W) and the last phenogram represents all granulocytes (area V). Each "spot" represents the percentage of cells falling into the possible combination shown at the top of each column. The "spots" are actually contours, whereby the number of contours represents the number of cells present. (Actual numbers are recorded in the saved phenogram file.) For each phenogram, the four columns are from left to right: dual negatives (−−), PE negative, FITC positive (−+), PE positive, FITC negative (+−), and finally dual positive cells (++). The Y axis is actually the TIP parameter and therefore represents from the bottom to the top each tube run in the assay (1–15). While the axes display 0–1,024 channels, this is a function of the instrument display software. Since the instrument was not designed with the notion of a parameter such as TIP, it cannot readily display it as the numbers 1–15. However, the flow cytometer identifies the TIP parameter as it would for any linear variable and displays it accordingly. The viewer must know what the order of the panel of antibody combinations is (See Table 1 to interpret the phenogram.)

The **bottom left** display in this figure shows the light scatter for the entire panel of tubes run in this set. Three gating regions are indicated: X (lymphocytes), W (monocytes), and V (neutrophils). Below the phenograms are some of the possible resultant light scatter displays from the gating regions drawn on the first phenogram. Region T is the $CD14^-/CD45^+$ region which should include all leukocytes except the monocytes (**second display, bottom row**). Region H is the $CD14^+/CD45^+$ region which should be positive for monocytes only as indicated by the resultant back-gated scatter display (**last display, bottom row**). What may not be totally obvious from this example is that the phenogram represents all possible fluorescence combinations for each cell type. A gating region drawn on a phenogram (2 are described) is capable of showing ANY combination of correlated variables for these cells. This means that fluorescence or scatter profiles (or combinations of both) can be displayed as 1 or 2 variables. No other gating is necessary because this has already be accomplished in the phenogram itself.

picture of the cross-reactivity of antibodies. Figure 2 shows one way in which the overview display can be used. In this figure, the coplot representation is juxtaposed with the overview. Thus, the overview display can provide both quantitative (each distribution can be gated and analyzed from the overview itself) as well as qualitative information (intensity information can be seen without the overlap of coplot presentations). One example of how the techniques presented were used to identify an antibody which presented a binding pattern inconsistent with its accepted phenotype is shown in Figure 2. CD16 antibodies were obtained from two different sources. By observing the binding patterns of the two CD16 antibodies on all cell types, it became obvious that one was not CD16. There were several reasons for this, one being the reduced intensity of the staining for neutrophils (see Fig. 2 CD16*). The second observation was the different patterns of binding on both lymphocytes and monocytes.

The different binding patterns can be seen by looking at the overview displays in Figure 3 rows 9 and 10 from the bottom. Figure 3 is a screen shot of a single analysis from the Elite workstation showing an overview of the entire panel as univariate fluorescence his-

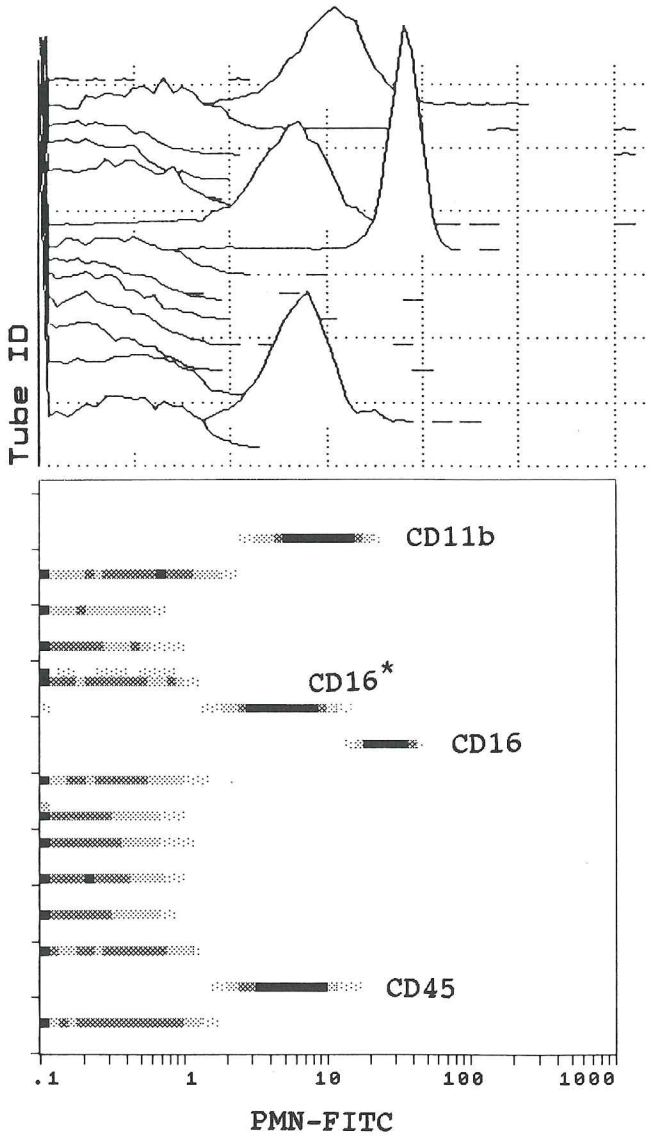


FIG. 2. This figure shows the *overview* display and *coplot* histograms in juxtaposition so that the overview concept can be appreciated. In this example are shown all of the patterns of fluorescence for just PMN, and then only for those antibodies conjugated to FITC. An identical PMN profile of PE-conjugated antibodies could be made by replacing the x axis variable with PE rather than FITC. The top figure is a regular coplot presentation for each marker. (See Table 1 for the markers.) Below is the identical information shown using the overview presentation. The intensity of the pattern indicates the cell number. Therefore, darker areas indicate more cells, lighter areas less cells. Each of the 4 strongly positive monoclonal antibodies in the upper figure is represented by dark lines in the lower figure. These displays can be individually gated to evaluate a specific tube number or even a specific component of the display. Looking at the 2 different CD16 histograms, it is clear that the fluorescence intensity of one (CD16*) is lower than the other. This marker was originally purchased as CD16 from Gentrack, but subsequently shown to be CD58. By comparing the cross-reactivity of these monoclonal antibodies using the techniques described in this paper, the different binding patterns on several cell populations can be determined rapidly. It is also possible to place a gate around any section of any distribution on this display and determine any required statistics.

tograms for PE and FITC fluorochromes. The *overview* plot describes each tube of the panel in terms of the degree of fluorescence (4 decade log scale). This presentation requires two displays to present the entire patient phenotype for a 2 color immunofluorescence assay. The advantages of the *overview* display are that both negative and positive labelled cells are visible and can be compared directly with controls. Further, it is possible to compare different tubes that contain one common antibody more easily. Several examples of this are shown in Figure 3. One is in the top right display for lymphocyte-FITC antibodies where the CD4 markers of tubes 5 and 6 or the CD8 from tubes 4 and 7 are directly compared.

Figure 4 is another screen shot showing coplot representations of all antibodies (Table 1) for each cell type (lymphocytes, monocytes, and neutrophils). This type of presentation is useful primarily for qualitative application of the marker intensities. Because all of the data for the entire set of tubes is in a single file, this figure was produced by a single operation taking less than a minute.

To produce statistical information for any of the analyses presented, there are several possibilities. Normal gates can be placed on any histogram or plot and statistics such as mean channel number, percentages, cv, and skewness are provided directly. Overview displays can be gated using traditional gates. For fully automated analysis of the overview displays, new software programs must be developed. Phenograms can be converted into numeric tables and imported into spreadsheet or database programs directly.

## DISCUSSION

The phenogram representation of multiparametric flow cytometry data is an innovative technique which, to some extent, solves a major data analysis problem that has plagued flow cytometry for many years (1). It is a significant break with the traditional data collection, analysis, and presentation techniques used in immunophenotyping. Used as a computer data file, the entire patient phenotype can be reduced to three small displays which can represent all leukocytes for the patient (in this case lymphocytes, monocytes, and granulocytes). The phenogram also provides a pattern useful for future development of expert systems in this complex area of immunopathology. One reason for this is that the phenogram itself, when saved as a computer file, contains a representation of the patient's phenotype in numeric form. Development of these applications is currently under way in our laboratory.

Of particular interest in this study was the cross-reactivity of some commonly used phenotypic markers used in clinical and research laboratories. Concern over the use of light scatter gating has resulted in development of backgating concepts that may assist in determining monocyte contamination in lymphocyte gates (8). While these techniques are now considered critical in clinical evaluation of immunophenotyping

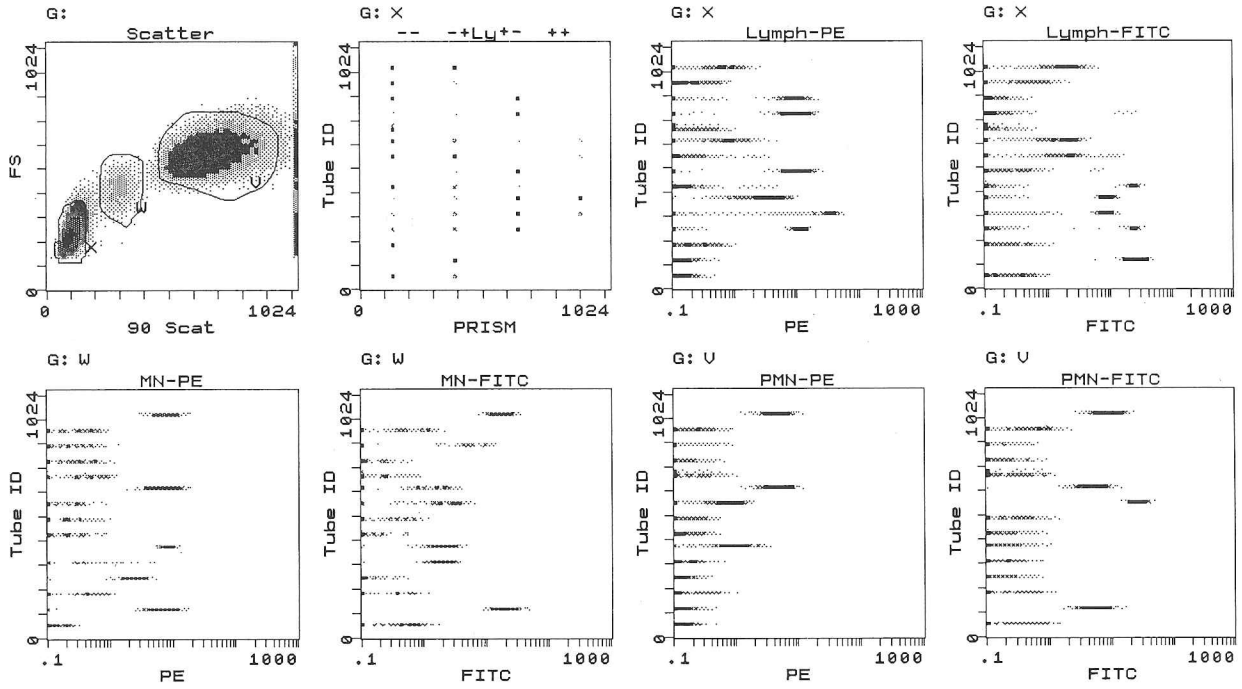


FIG. 3. The first plot shows the light scatter from which gating decisions were made. The next display shows the phenogram representing all possible 2 color combinations for the entire panel. The next 6 displays are overviews for each cell type [lymphocytes (lymph), monocytes (MN), and granulocytes (PMN)] and for each fluorochrome selected (PE and FITC). These are fluorescence intensity displays for each tube in the assay starting from tube one at the bottom to tube 15 at the top of each display. Above each display the gate region (G:) identifies the light scatter gate used: X-lymphocytes; W-monocytes; V-PMN. On the left of each set of two displays is the PE fluorochrome and on the right the FITC fluorochrome. It is possible to see all positive and negative cells for each antibody combination selected. These *overviews* allow a rapid qualitative and semi-quantitative observation of the cross-reactivity of each antibody for each cell type selected. Each overview is derived from a light scatter gated populations of cells. This entire figure is the result of a single rapid analysis and required less than a minute to complete including obtaining the printout and statistics of selected regions.

data, it is clearly not possible to include monoclonal antibodies such as CD45 and CD14 in every tube! Because of this common practice is to determine the appropriateness of scatter gates using this monoclonal antibody combination but then proceed to analyze samples based largely upon scatter characteristics (6). This is not unreasonable, since the scatter characteristics of multiple tubes from a single patient should be essentially identical, if prepared correctly. This, then, is the basis of the production of phenograms using scatter gating, since all of the quality control characteristics presently used for immunophenotyping are inherent in its structure.

With development of the phenogram, it is possible now to view the entire phenotype in one multidimensional display, enabling complex multivariate analysis previously possible only after exhaustive analysis. The phenogram displays are either hatched patterns, contours, or color coded displays. In each case the intensity of the pattern or its color reflects the percentage of cells. Therefore, the patterns produced indicate the presence or absence of a population of cells. The levels of significance can easily be altered to reflect a desired sensitivity.

The data shown in these phenograms clearly demonstrate instances when an antibody identifies cells from more than one light scatter gated population. Using multiple cell phenograms the chances of an incorrect percentage of positive cells (because of incorrect gating) is reduced because the control is always visible. This information is usually not detected until a post collection evaluation of the data is performed. Further, it can only be corrected if list-mode data are accumulated; otherwise the samples must be re-run on the cytometer. If the phenogram is collected, it can be displayed during acquisition of samples, allowing the operator to detect poor gating by observing particular "spots" of interest. Post-data analysis and interpretation are greatly enhanced by use of the phenogram, which lends itself to rapid pattern recognition techniques.

The addition of the overview plots enhances the ability to detect cross-reacting antibodies also. For each cell type, multiple overview displays can be displayed showing each fluorescence probe. In the present example a two color assay was performed to produce PE and FITC histograms. Each tube in the assay was run consecutively and therefore, since all the tubes were displayed beside one another, a rapid comparison of each color marker was possible. When

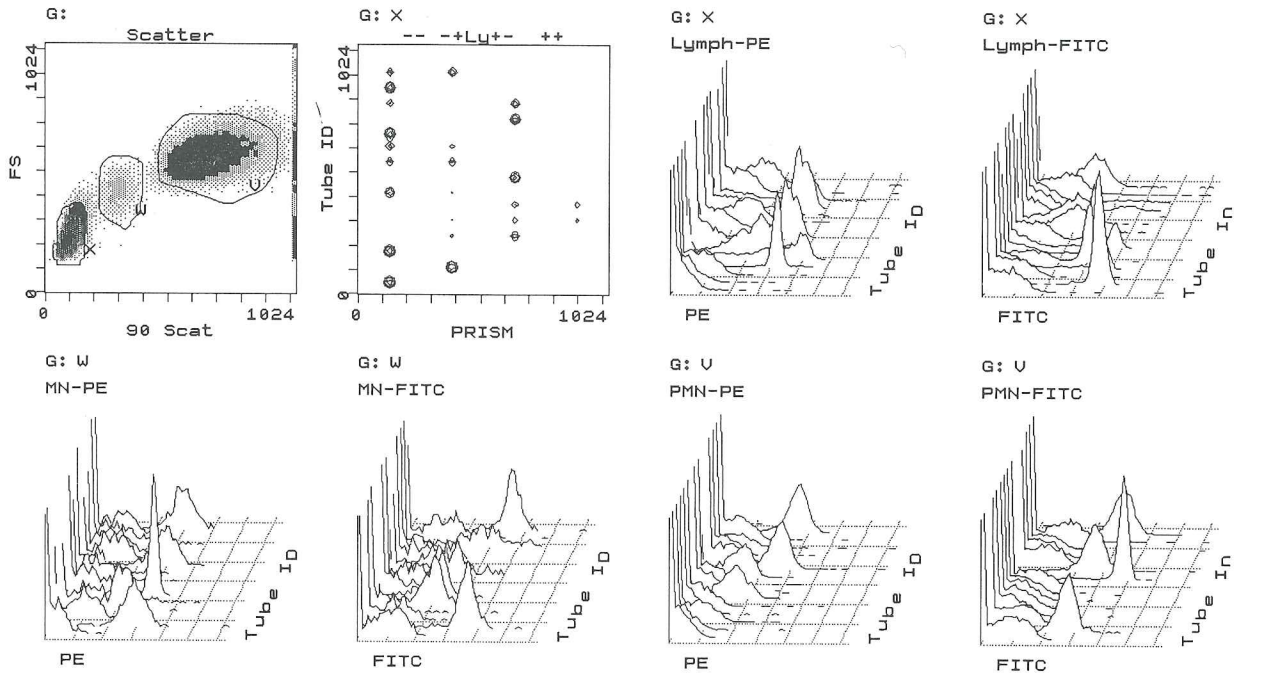


FIG. 4. This is a series of coplot histograms showing each antibody marker as it appears on each cell type selected. Three cell types were selected for analysis: lymphocytes, monocytes, and granulocytes from peripheral blood. The coplot provides an alternative method for displaying the overview plots. This representation provides a rapid qualitative analysis of antibody markers on a patient. This overview allows particular markers (such as shown in Fig. 2) to be evaluated across all cell types in the assay.

comparing antibodies on different cell types, it is only necessary to plot each of the overview displays from these populations to determine the cross-reactivity for each antibody.

One of the valuable considerations of this technique is the speed with which the analyses take place. Whereas normal analysis of flow cytometric data requires laborious manipulation of many files and histograms, the TIP technique allows any number of tubes to be treated as a single set, and therefore analyzed in a single step. There are many instances in interpretation of clinical or research data where it is desirable and necessary to display histograms from several different files simultaneously to achieve a diagnosis or solution (4). When this is necessary, a specific profile can be established as any combination of 8 uni or bivariate plots that can be displayed on the screen simultaneously. Once profiles of interest are established they can be rapidly applied to the data file. The time taken to produce any such analyses never exceeds 1 min, regardless of the complexity of the analysis or the number of histograms presented.

The technique presented is a valuable tool for evaluation of cross-reacting antibodies, improper gating, or simultaneous analysis of multiple cell populations. We believe that a variety of uses for the phenogram will become apparent, particularly as more complex experimental designs are attempted in flow cytometry such as 3, 4, and 5 color simultaneous measurements. The phenogram is capable of handling 3, 4, or 5 color data equally well as the 2 color data presented in this communication.

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