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# Comparison between Dual and Tri-Colour Reagents for the Analysis of Lymphocyte Subsets

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**SUMMARY** **The aim of this project was to compare dual and tri-colour reagents for lymphocyte immunophenotyping. A total of 37 patient and normal specimens were immunophenotyped concurrently with the following mean values (% dual *vs* tri-colour) : CD3 (69.4 *vs* 68.3) CD4 (24.0 *vs* 24.2) and CD19 (13.9 *vs* 12.6). A comparison of the results obtained using the paired *t* test showed that there were no significant differences for cells expressing CD3, CD4 and CD19. However, there was a significant difference in the NK (18.3 *vs* 16.3) cell component. A major advantage in using 3 colour immunophenotyping is the ability to analyse specimens that cannot be analysed using dual colour reagents due to debris or contamination of the gate with non-lymphocytic cells.**

Immunophenotyping of lymphocyte subsets is necessary for the diagnosis of some primary and secondary immunological disorders. Although viral loads are being investigated for their potential as prognostic tool,[1] the absolute CD4 count is still crucial in HIV infection as an indicator for disease progression, treatment initiation points and the diagnosis of AIDS.[2-4] This parameter is usually measured at three to six month intervals. As such, accuracy and reproducibility of the results are important. Methods to determine absolute CD4 counts include light microscopy, fluorescent microscopy and the ELISA. The use of light and fluorescent microscopy requires the identification of lymphocytes based on morphology and the expression of CD antigens on the cell surface. This approach suffers from two limitations. Firstly, morphological characteristics may be ambiguous to the untrained eye especially if the patient has elevated white blood cells, is leucopenic or at later stages of HIV infection. Secondly, the CD4 and CD8 markers which are used for subset enumeration may be found on more than one cell type.[5,6]

In order to overcome these limitations, dual reagents are commonly used for enumeration. This reduces ambiguity and increases accuracy. We have, however, found that a second sample of blood has to be requested for up to 10% of specimens received as they fail to meet the criteria for a valid analysis. In order to reduce the repeat rate as well as improve accuracy and reproducibility, some laboratories are assessing the use of three colour reagents. In this paper, we compare the current dual colour method with a tri-colour method for enumerating lymphocyte subsets and discuss the possible pitfalls in adopting a three colour protocol.

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## MATERIALS AND METHODS

### Blood

Blood sent to the Division of Immunology, Institute for Medical Research for lymphocyte phenotyping was used in this study. Analysis of the accompanying request forms showed that these included 13 patients seropositive for HIV, 5 suspected primary immunodeficiencies, 10 controls and 5 with unstated reasons for immunological investigations. All blood was kept at room temperature and processed within six hours of venesection. Approximately 2 ml of blood in vials containing ethylenediaminetetraacetic acid (EDTA) and kept at ambient temperature was sent to the Institute for Medical Research. The total white blood cell count (TWBC), percentage lymphocytes and absolute number of lympocytes were determined using a haematological cell counter (Model T540, Coulter Co, USA).

### Monoclonal antibodies

Monoclonal antibodies used for the enumeration of T and B cell subsets were selected according to the recommendations of the US National Committee for Clinical Laboratory Standards.[7,8] Selection of monoclonal antibodies was based on the need to correctly identify all lymphocyte subsets, as CD antigens may be found on more than one population of cells. The following monoclonal antibodies were used in each panel: Anti-CD45 fluorescein isothiocyanate (FITC)/anti-CD14 phycoerythrin (to identify the lymphocyte population), immunoglobulin G1 FITC/IgG1 PE (negative isotype control), anti-CD3 FITC/anti-CD19 PE (T and B cells), anti-CD3 FITC/anti-CD4 PE (T helper cells), anti-CD3 FITC/anti-CD8 PE (T suppressor/cytotoxic cells), anti-CD3 FITC/anti-CD16+ anti-CD 56PE (NK cells), anti-CD3 FITC/anti-HLA-DR (activated T cells), anti-CD3 FITC/anti-CD19 PE/anti-CD45 PerCP, anti-CD3 FITC/anti-CD4 PE/anti-CD45, anti-CD4 FITC/anti-CD8 PE/anti-CD3 PerCP, anti-CD3 FITC/anti-CD16+anti-CD56 PE/anti-CD45 PerCP,IgG gamma 1 FITC/IgG gamma1PE/anti-CD45 PerCP. All monoclonal antibodies were purchased from Becton Dickinson (Mountain View, CA).

### Staining of cells

One-tenth millilitre of blood is carefully placed in a 12x75 mm. round bottomed polystyrene tube (Falcon 2052, Becton Dickinson, Lincoln Park, New Jersey, USA) Twenty microlitres of the appropriate antibody conjugate was added to the blood and mixed thoroughly. The tubes were then incubated for 15 minutes in the dark at 4°C followed by lysis of red blood cells using 2 ml of a 1:10 dilution of FACSLYSE (Becton Dickinson, Mountain View, CA) in distilled water. The tubes were incubated for not more than 10 minutes at room temperature and then centrifuged at 300×g for 5 minutes. The cells were then washed once with phoshate buffered saline before being resuspended in PBS pH 7.2 containing 1% formaldehyde. Cells were kept overnight before analysis in a FACSCAN.

### Analysis of data

Cells were analysed in a FACSCAN using Simulset software (Becton Dickinson, Mountain View, CA). The absolute lymphocyte numbers obtained from the haematological cell counter for each patient were entered into the database. Approximately 2,000 lymphocytes were analysed in each tube. Results obtained were in percentage and absolute number of the various subsets tested. In order to ensure validity of the analysis, the data were only included if (a)% T cells+% B cells+% NK cells= 90-110 %, (b) 90% or more of the total lymphocyte population was analysed, (c) more than 90% lymphocytes were in the analysis gate and (d) the forms accompanying the specimens were completed legibly and correctly. The three colour specimens were analysed using Lysys II software. List mode data for 10,000 cells were collected for analysis. Analysis was carried out by gating on the CD45 PerCP *vs* SSC display. Gated cells were then analysed using the following displays:CD3/CD19, CD3/CD4, CD3/CD16.

### Statistical analysis

Statistical analysis was carried out using Statistical Package for Social Science (SPSS) for Windows as well as Lotus 1-2-3 Release 5.0 for Windows. Results were compared using the paired *t* test because the same specimen was tested by the two methods being compared.

## RESULTS

Blood from 37 patients and controls was immunophenotyped using dual and three colour reagents. All specimens were processed simultaneously. In five cases, unsatisfactory results were obtained using the dual colour reagents largely due to the lymphosum being out of range, the percentage lymphocytes in the gate being below 90% or the software being unable to locate the lymphocyte gate. These specimens were excluded from the comparison. The mean, standard deviation and range for CD3, CD4 CD19, NK and HLA-DR cells were calculated for each reagent and is shown in Table 1. The range was obtained using the 10 and 90 percentile values. In order to compare the values for CD3, CD4, CD19 and NK cells obtained using the two methods, the paired *t* test was used. There were no significant differences for CD3, CD4 and CD19. However, mean values obtained for NK cells were significantly different using the two methods, ($p$=0.001) Satisfactory results could not be obtained for five patients using dual colour reagents. However, when the same specimens were processed using three colour reagents, results that met the current guideline could be obtained ( Table 2).

**Table 1.** Comparison of results obtained using two and three colour reagents. The range represents the 10 and 90 percentile points.

| CD Antigen | Reagents | Mean | SD | Range | p value paired t test |
|---|---|---|---|---|---|
| CD3 | Dual | 69.4 | 12.9 | 52.3-86.2 | 0.175 |
| | Tri | 68.3 | 13.2 | 54.3-86.2 | |
| CD4 | Dural | 24.0 | 13.3 | 3.3-41.5 | 0.635 |
| | Tri | 24.2 | 13.4 | 3.0-38.7 | |
| CD19 | Dual | 13.9 | 7.5 | 6.0-24.5 | 0.023 |
| | Tri | 12.6 | 8.7 | 3.0-22.0 | |
| NK | Dual | 18.3 | 8.8 | 8.3-31.0 | 0.001 |
| | Tri | 16.3 | 8.4 | 6.3-26.0 | |
| HLA-DR | Dual | 25.9 | 13.5 | 10.0-46.0 | |
| | Tri | nd | nd | nd | |

SD = Standard deviation
nd = Not done

**Table 2.** Results of specimens which could not be analysed by the two-colour protocol were analysed by the three-colour protocol.

| Patient No. | CD3 | CD19 | CD4 | NK | T+B+NK |
|---|---|---|---|---|---|
| 08-2607 | 74.3 | 8.0 | 19.0 | 14.0 | 96.3 |
| 08-2485 | 57.3 | 30.0 | 20.0 | 11.0 | 98.3 |
| 08-2604 | 45.0 | 34.0 | 33.0 | 16.0 | 95.0 |
| 08-2419 | 74.3 | 3.0 | 6.0 | 17.0 | 94.3 |

## DISCUSSION

The availability of dyes with spectral emissions that do not overlap those currently available when excited at 488 mm enabled the introduction of three colour reagents for immunophenotyping by flow cytometry. Both single tube and multi-tube assays using three colour reagents have been reported.[9-13] The main advantages of using a single tube three colour assay is the reduction of cost and processing time. Furthermore, there is no necessity to determine the lymphosum because, by definition, only cells that meet the predetermined criteria are being analysed. The problem of non-lymphocytes being included in the analysis does not arise. The main drawback however, is that the software is not automated. The results obtained using the two methods were compared. There were no statistical differences between results obtained using the two methods for CD3, CD19 and CD4. Three colour reagents were not used to determine the percentage of T cells showing HLA-DR expression. Unsurprisingly, the expression of HLA-DR was much higher than reported for the normal Malaysian population[14] because the population studied here included a number of

patients with infection. The NK cell percentage in this population is lower compared to data reported earlier on the normal Malaysian population.[15] It is, however, higher compared to the Caucasian population. Possible reasons for this are discussed elsewhere.[15] There is a significant difference in NK percentages obtained using the two methods. The three colour methods gives a lower percentage compared to the dual colour methods. The reason for this is not clear. It is possible that the CD45/SSC gating may not be sufficient to include all NK cells in the analysis. The FSC/SSC gate combined with the CD45/CD14 gate used for dual colour analysis may be able to capture all the NK cells.

It is important to use the correct combination of reagents in order to obtain the correct results. We feel that it would be inadvisable to use a CD3/CD4/CD8 combination because it overestimates the CD4 and CD8 component. This occurs because analysis using a gate determined on CD3/SSC uses CD3 as a denominator unlike a gate using CD45/SSC which using CD45 comprising B cells, T cells and NK cells as a denominator. Thus the CD3 and CD4 percentages using the former combinations are overestimated (submitted).

In a small proportion of cases results cannot be obtained using the dual colour reagents. Specimens are usually rejected because they do not fall within the quality control guidelines. Reasons for rejection of such specimens include excessive amounts of debris, lymphosum less than 90 or more than 110, or fewer than 90% lymphocytes in the gate. The three colour protocol overcomes the limitations of gate characteristics by identifying cells solely on the expression of surface markers and not by cell size or morphology. Thus, one of the major advantages of using three colour reagents is the drastic reduction of rejected specimens. Software available for analysis of three colour specimens is not automated. Thus, it may not be expedient to use this method for routine analysis. However, it is sometimes difficult to determine in advance the difficulty of analysing a particular specimen. In order to overcome this one can either add CD45 PerCP to all the tubes in the dual colour panel or one can reprocess the blood.

It is obvious that there are advantages in using a three colour protocol over the dual colour protocol. However, it should be noted that the cost of the assay and the present unavailability of autmated software must be taken into consideration before such a test is introduced.

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