COMPARISON BETWEEN FLOWCYTOMETRY AND IMMUNOPEROXIDASE STAINING FOR THE ENUMERATION OF LYMPHOCYTE SUBSETS

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ABSTRACT

Immunoperoxidase staining was compared with flowcytometry for the enumeration of lymphocyte subsets. The percentages obtained for peripheral blood lymphocytes using immunoperoxidase (CD3=76 CD4=50.5, CD8=27.9, B=10.7 CD4/CD8=1.8) differed significantly from those obtained by flowcytometry (CD3=65.7 CD4=39.4, CD8=25.6, B=16.7, HLA DR=11.9 CD4/ CD8=1.54) for certain subsets (CD3, CD4, B). There was no significant difference in lymphocyte subsets between children and adults using the same method. These differences are probably due to the different methods used to prepare lymphocytes for analysis. Other factors that should also be considered are the presence of CD4 antigen on monocytes and CD8 on natural killer cells.

Keywords: Immunoophenotyping, lymphocyte subsets, Immunoperoxidase, flowcytometry.

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INTRODUCTION

Monoclonal antibodies against cell surface antigens on lymphocytes may be used for the identification and characterisation of lymphocyte subsets[1]. Some markers are associated with the functional characteristics of lymphocytes whilst the role of others remain unclear. As the percentage of these various subsets are quite characteristic, their measurement offers a means of identifying perturbations. It is now common practice to use lymphocyte subset enumeration to identify and diagnose primary and secondary immunodeficiencies and haematological malignancies.

Enumeration of lymphocytes has been carried out in our laboratory since 1987. We have been using the immunoperoxidase method for enumeration and this involves reading slides with a light microscope. We have recently used the flowcytometer to carry out this task. In flowcytometry, cells are stained with a fluorescent conjugated monoclonal antibody. The amount of fluorescence on cells is then analysed by the cytometer to determine positive staining. This paper describes some observations made after reviewing the results of immunoperoxidase staining and examines the reasons for the discrepancies on comparing the results obtained with flowcytometry.

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MATERIALS AND METHODS

Blood

Peripheral blood was obtained from donors at the Blood Bank, General Hospital, Kuala Lumpur. Blood was also obtained from potential kidney donors at the National Tissue Typing Laboratory, IMR, Kuala Lumpur. Blood from children below the age of twelve was supplied as age matched controls with patient specimens. Approximately 10 ml of blood was collected in EDTA by venipuncture. It was transported to the laboratory at ambient temperature. Analysis was performed within 6 hours of specimen collection.

Monoclonal antibodies

Flowcytometry

The panel of antibodies were either Flouresecein Isothiocynate or Phycoerythrin conjugated monoclonal antibodies so that they could be used for dual-colour analysis (Becton Dickinson, Mountain View, CA). The panel consisted of the following reagents: Anti-CD 14 PE/Anti-CD45 FITC, IgG1 FITC/IgG2 PE (negative control), Anti-CD4 FITC/Anti-CD 8 PE (helper/ suppressor ratio), Anti-CD3 FITC/Anti-CD 19 PE (T and B cells), Anti-CD3 FITC/Anti HLA DR PE (Activated T cells).

Immunoperoxidase staining

The following antibodies from Ortho Diagnostic System Inc. were used for the determination of lymphocyte subsets: OKT 3 (Total T cells), OKT 4 (T Helper cells), OKT 8 (T suppressor/ cytolytic cells) and OKB 7 (B cells).

Staining and analysis of cells

Flowcytometry

0.1 ml of blood is carefully placed in a 12 x 75 mm round bottomed polystyrene tube (Falcon 2052, Becton Dickinson, Luncoln Park, New Jersey, USA). Twenty microlitres of antibody conjugate is then added to the blood and it is mixed throughly. The blood is incubated at room temperature in the dark for 10 minutes. It is then placed in a Coulter Q-Prep for the thirty five second cycle to lyse red blood cells. Lysis is carried out by the addition of the following reagents: 70 microlitres of Immunoprep A (containing Formic acid), 32 microlitres of Immunoprep B (containing sodium carbonate (6.0 g/lit), sodium chloride (14.5 g/lit) and sodium sulphate

(31.3 g/lit)) and 14 microlitres of Immunoprep C (containing 10 g/lit of paraformaldehyde). The tubes are then centrifuged at 200g for 10 minutes, the supernatant is discarded and 0.5 ml of PBS pH 7.2 is added and the cells are resuspended ready for analysis.

Analysis of lymphocytes was done using the FACSCAN and Simulset software (Becton Dickinson, Mountain View, CA). Gates on the lymphocyte population were set automatically by the software. Dimly stained CD 8 cells were excluded from the count by manually moving the gates. The amount of contamination by granulocytes and monocytes in the gate was also provided by the instrument. 2,000 lymphocytes were analysed for fluorescence.

Immunoperoxidase staining

Ten millilitres of blood is collected in Venoject tubes (Terumo Corporation, Japan) containing 200 U of heparin (Flow Labs, UK). It is centrifuged at 200g for 10 minutes, the supernatant is discarded and the cells are washed once with PBS (pH 7.2). The pellet is resuspended with an equal volume of PBS and the suspension is carefully layered on Ficoll-Hypaque (Pharmacia AB, Sweden) at a 2:1 ratio of blood to Ficoll. The tubes are spun for 10 minutes at 300g and the white blood cell layer is carefully removed using a Pastuer pipette. The cells are then washed twice with PBS. The pellet is resuspended in 1ml RPMI 1640 (Flow Labs, UK) with 10% FCS (Flow Labs, Scotland) and counted. The concentration is adjusted to 2 x 10^6 cells per ml. Ten microlitres of the appropriate monoclonal antibody is added to 90 microlitres of the cell suspension. The cells are then vortexed and incubated for half an hour in ice. Two of cold PBS with 2% BSA is then added to the tubes. The resulting suspension is centrifuged for 5 minutes at 200g. The supernatant is then discarded and 1 ml of 0.1% glutaraldehyde is added to the suspension followed by incubation for 10 minutes at room temperature. The suspension is washed twice in PBS (pH 7.2) after centrifugation for 5 minutes at 200g. A smear is made on a microscope slide which is dried overnight. The slides are then stained as follows: They are incubated for 20 minutes with 1 - 3 drops of mouse anti-immunoglobulin. The slide is then washed in PBS and 1 - 3 drops of peroxidase labelled mouse immunoglobulin is placed on the slide and further incubated for 20 minutes. After washing with PBS a few drops of substrate solution (amino-ethylcarbazole) is added. The slides are then incubated for 20 minutes. They are washed with distilled water and counterstained with Mayer's hematoxylin (BDH Chemicals, Poople, UK) for 1 minute before being flooded with ammonia water and washed with tap water. They are then wet-mounted with buffered glycerol and approximately 300 lymphocytes are counted for each slide. Care is taken to correctly identify the lymphocytes. Positive cells are stained reddish-brown.

STATISTICAL ANALYSIS

Statistical comparisons were made using the Student t test. Ranges given are the interquartile ranges (IQR).

RESULTS

Enumeration of lymphocyte subsets in adults using immunoperoxidase

Using the immunoperoxidase method, lymphocytes from 103 blood donors were analysed to establish the means and ranges of lymphocytes subsets in the normal population. The OKT 3 monoclonal antibody was used to estimate the number of T cells, while OKB 7 was used to estimate the number of B cells. Monoclonal antibodies against CD4 (OKT 4) and CD8 (OKT 8) were used to determine the helper and suppressor/ cytolytic T cell ratio. The results obtained are shown in Table I. Seventy-six percent of the lymphocytes were shown to be T cells and 10.7 percent were shown to be B cells; 50.5 and 27.9 percent of lymphocytes were shown to be CD4 and CD8 cells respectively. This gave a CD4/CD8 ratio of 1.8.

Comparison of flowcytometry with immunoperoxidase

Using flowcytometry, 26 blood donors were immunophenotyped to establish normal values for the lymphocyte subsets. The results obtained show that the mean percentage of T and B cells was 65.7 and 16.5 percent respectively. The percentage of CD4 and CD8 cells was 39.4 and 25.6 respectively giving a CD4/CD8 ratio of 1.54. The mean percentage of activated T cells was 11.9 percent. Only brightly stained CD 8 cells were counted. Comparison of the results in Table I and Table II indicate significant differences for some subpopulations. The values for CD3 and CD4 cells using flowcytometry is significantly lower compared to light microscopy ($p<0.0001$). The reverse is true for B cells ($p<0.0001$). There were no significant differences in results obtained for CD8 cells or CD4/CD8 ratios. In 8 cases, analysis was carried out by both methods. Statistical comparison using the paired t-test showed results similar to that obtained above. (CD3, CD4, and B: $P<0.05$; CD8, CD4/CD8: not significant).

Table I. Enumeration of lymphocyte subsets in adults using immunoperoxidase (n=103).

CD Antigen	CD3	CD4	CD8	B	CD4/CD8
Mean	76.0	50.5	27.9	10.7	1.8
S.D.	7.1	6.2	5.4	3.0	0.35
Range	71-81	46-55	25-31	9-12	1.64-2.1

CD4/CD8 is expressed as a ratio.
All other results are expressed in percentage.

Table II. Enumeration of lymphocyte subsets in adults by flowcytometry

CD Antigen	CD3	CD4	CD8	B	DR	CD4/ CD8
Mean	65.7**	39.4**	25.6	16.5**	11.9	1.54
S.D.	8.4	5.3	4.8	4.4	5.8	0.43
Range	72-59	34-43	22.7-27.5	11-19	11.5-19	1.21-1.87

(** = Means are compared to data in Table I; $p<0.0001$) (n-=26).
CD4/CD8 is expressed as a ratio.
All other results are expressed in percentage.

Enumeration of lymphocyte subsets in children using immunoperoxidase

A total of nineteen children ranging in age from 1 to 12 (Table IIIa) were phenotyped using the immunoperoxidase method. The lymphocytes were typed with OKT 3 (Total T cells), OKT4 (Helper T cells), OKT 8 (Supresson/Cytolytic T cells) and OKB7 (Total B cells) monoclonal antibodies. The results, shown in Table IIIb revealed that the percentage of total T and B cells was 73.4 and 12.7 respectively. The values for helper T and Suppressor/cytolytic T cells was 46.7 and 26.8 percent respectively which gave a CD 4/CD8 ratio of 1.8.

Table IIIa. Age distribution of children used in analysis

Age	1	2	3	4	5	6	7	8	9	10	11	12
No.	7	2	1	1	0	3	1	1	1	1	0	1

Comparison of lymphocyte enumeration in children and adults

Establishment of normal values in children (Table IIIb) and comparison of values obtained with adults (Table I) using the same method revealed that there were no significant differences between the two groups ($p<0.0001$ for all subsets).

Table IIIb. Enumeration of lymphocyte subsets in children using immunoperoxidase (n=19).

CD Antigen	CD3	CD4	CD8	B	CD4/CD8
Mean	73.4	46.7	26.8	12.7	1.8
S.D.	7.6	5.6	5.2	4.3	0.4
Range	69-79	44-51	23-32	10-15	1.52-2.0

CD4/CD8 is expressed as a ratio.
All other results are expressed in percentage.

DISCUSSION

The analysis of lymphocyte subset enumerations carried out over the past 3 years by the immunoperoxidase method, and more recently by flowcytometry, has revealed a number of interesting observations. The total number of T lymphocytes was determined by using a monoclonal antibody against the CD3 antigen[2]. This is a 19 kD protein present on all peripheral T lymphocytes as well as the Purkinje cells in the cerebellum of a number of species[3]. Although the majority of thymocytes express both CD4 and CD8 markers, they are expressed on mutually exclusive populations of mature T cells in peripheral blood[4]. The CD4 antigen is a 55kD glycoprotein[5] that is associated with recognition of antigens in the context of MHC class II molecule. CD8 cells are associated with recognition of antigen in association with Class I molecule. It has been suggested that CD4 cells may be further subdivided based on their lymphokine secretion profiles. Some evidence seems to indicate that this may be possible using the relative expression of CD45 antigen[6,7].

Table I shows the percentage of T cells (76.0), B cells (10.7), helper cells (50.0) and suppressor/cytolytic cells (27.9) in normal donors using immunoperoxidase. This is consistent with other findings utilising similar methodology[8]. The markers above and HLA-DR were also analysed by flowcytometry. A comparison of the subset populations arrived at by these two methods indicates significant differences in results obtained for CD3, CD4, and B cells. There could be a number of reasons for this. Human T and B cell proportions are significantly influenced by the methods used to isolate lymphocytes[9]. Their separation on density gradient media may result in the selective loss of certain lymphocyte subpopulations. Although this effect may not be pronounced in normal specimens, it may cause a significant difference in results obtained from the blood of leucopenic patients. If all subpopulations are to be represented, then blood should be prepared by whole blood lysis. These findings also reinforce the notion that established methods should not be modified without further investigation. There are some markers which are expressed on other lymphocytes. This may be a source of erroneous results although it can be controlled by carefully choosing the panel of antibodies used in analysis. For example, CD4 antigen is also present in low concentrations on monocytes and in the cytoplasm of monocytes and macrophages[10]. It is very important to identify and only count the lymphocytes. CD4+ monocytes as well as cells with cytoplasmic staining should be identified and excluded. Natural killer cells also constitute an important subpopulation when intepreting data on the enumeration of lymphocyte subsets. They comprise 10% of peripheral blood lymphocytes[11]. They are large granular lymphocytes that do not express CD3 but are capable of lysing target cells without MHC restriction or prior immunisation[12]. It has been suggested that the panel should include an anti-CD16 or anti Leu-19 monoclonal to measure the NK subset[13]. Some NK cells express low levels of CD 8 antigen[14]. These cells should be identified and excluded from the CD8+ count.

Immunophenotyping revealed that there were no significant differences in lymphocyte subset populations when comparing children and abults (Table II, Table IIIb). This seems to indicate that age-matched controls are not absolutely necessary when carrying out the test for children.

Investigators continue to identify markers that may assist in the diagnosis and prognosis of disease. Their careful evaluation may also point the way to understanding the pathogenesis of certain disorders. It is therefore important to continually test new markers with a wide range of diseases.

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