Direct immunomagnetic quantification of lymphocyte subsets in blood

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SUMMARY
A method is described where superparamagnetic polymer microspheres coated with monoclonal antibodies (MoAb) are used for the direct and fast quantification of the absolute number of cells of various lymphocyte subsets in blood. Blood samples were incubated with microspheres coated with a subset specific MoAb. Using a magnet the microsphere-rosetted cells were isolated and washed. Following lysis of the cell walls to detach the microspheres, the cell nuclei were stained with acridine orange and counted in a haemocytometer using an immunofluorescence microscope. With MoAb specific for CD2, CD4, CD8 and CD19, reproducible absolute counts of the corresponding lymphocyte subsets were obtained which correlated closely with those obtained by an indirect quantification method.

Keywords immunomagnetic quantification lymphocyte subsets

INTRODUCTION
Quantification of lymphocyte subsets is an important routine test in clinical immunology. Most methods are based on the initial separation of peripheral blood mononuclear cells (PBMC) by gradient centrifugation (Boyum, 1968), followed by the identification and estimation of lymphocyte subsets as fractions of the total PBMC by rosetting techniques (Pellegrino, Ferrone & Theophilopoulos, 1976) or flow cytometry (reviewed in Renzi & Ginnis, 1987). The absolute number of cells of a subset can be calculated provided the total leucocyte count and the fraction of mononuclear cells or lymphocytes as a percentage of total leucocytes are known.

Recently immunomagnetic (IM) isolation of fractions of cell subsets directly from blood has been described (Vartdal et al., 1986; Funderud et al., 1987). The present paper describes the IM isolation directly from blood of all the cells of various lymphocyte subsets and their quantification using a haemocytometer. The method is simple and fast and gives reproducible results.

MATERIALS AND METHODS

Donors
Blood from healthy laboratory personnel was collected in Vacutainer tubes containing EDTA (Becton Dickinson Vacutainer Systems Europe, Grenoble, France). One millilitre of blood was required for each subset counted immunomagnetically. Unless otherwise stated, experiments were begun within 4 hours of venesection.

Monoclonal antibodies (MoAb)
An MoAb specific for the CD2 antigen (clone BMA 0111/IgG1) was purchased from Behringwerke Diagnostica, Marburg, FRG. An MoAb specific for the CD4 antigen (clone 66-1/IgM) was generously provided by Professor J. Hansen (Fred Hutchinson Cancer Centre, Seattle, WA, USA) while an MoAb specific for the CD8 antigen (clone ITI-5C2/IgM) has been produced in our laboratory (Gaudernack et al., 1986). Monoclonal antibodies specific for B cell antigens CD19 (clone AB1/IgM) and CD37 (clone HH1/IgG1) and for β2 microglobulin (clone E2-14/IgG) have been produced by S. Funderud (Melson et al., 1984; Smeland et al., 1986). Monoclonal antibodies OKT4, OKT8 and OKT11 were purchased from Ortho Pharmaceuticals (Raritan, NJ, USA). A mouse ascites containing an MoAb (clone 2F4/IgG) with no known specificity was used as a negative control.

Microspheres and coating procedures
The properties of IM microspheres (Dynabeads™ M-450, Dynal, Oslo, Norway) and details of coating procedures are described elsewhere (Funderud et al., 1987). Monoclonal antibodies used in this study were coated onto Dynabeads M-450, 50 mg/ml, in the following concentrations: BMA 0111; 200 μg/ml; 66-1, 300 μg/ml; ITI-5C2, 100 μg/ml; AB-1, 150 μg/ml.
Table 1. Volumes of ingredients (ml) making up the rosette suspension and the suspension of cell nuclei

<table>
<thead>
<tr>
<th>Cell subset assayed</th>
<th>CD2</th>
<th>CD4</th>
<th>CD8</th>
<th>CD19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosette suspension</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>1.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>PBS/0.3% BSA</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>M-450-MoAb*</td>
<td>0.6</td>
<td>0.5</td>
<td>0.25</td>
<td>0.2</td>
</tr>
<tr>
<td>Suspension of cell nuclei</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZA Pomin</td>
<td>0.1</td>
<td>0.1</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>PBS/0.3% BSA</td>
<td>1.8</td>
<td>1.8</td>
<td>0.9</td>
<td>0.425</td>
</tr>
<tr>
<td>Acridine orange</td>
<td>0.1</td>
<td>0.1</td>
<td>0.05</td>
<td>0.025</td>
</tr>
<tr>
<td>Total volume</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Microspheres coated either directly or indirectly with MoAb (see Materials and Methods) and resuspended to a concentration of 10 mg/ml.

Isolation and quantification of lymphocyte subsets

For the quantification of each lymphocyte subset 1 ml EDTA-blood was diluted with 2 ml of phosphate buffered saline containing 0.3% bovine serum albumin (PBS/BSA). The blood was kept at 2–8°C to avoid non-specific attachment of microspheres to phagocytic cells. Microspheres coated with MoAb specific for T cells (CD2, CD4 and CD8) or B cells (CD19) were added to the tubes, 35–50 microspheres per expected maximal number of target cells (Table 1), and the suspension mixed on a Rock-N-Roller at 2–8°C for 1 h. A cobalt-samarium magnet was then applied to the outside wall of the test tube for 2–3 min to collect rosetted cells and free microspheres onto the inside wall of the tube. The remaining blood/buffer solution was then pipetted off. Cells and microspheres were subsequently washed three times. Each time, cells and microspheres were resuspended in ample volumes of the PBS/BSA. The magnet was then applied to the outside of the tube for 30 s and the washing buffer finally removed. Having sucked off the buffer completely after the third washing cycle, cells and microspheres were flushed down from the side of the test tube with 50 µl or 100 µl of the detergent ZA Pomin (Coulter Electronics Ltd, Luton, Beds, England) to lyse the cell walls, thereby detaching the microspheres from the cells. PBS/BSA was added to make up a suitable volume (see Results, Method design), and the cell nuclei were stained with a final concentration of 1.5 µg/ml acridine orange (Sigma, St Louis, MO, USA) (Table 1). Throughout these procedures the cells were kept at 2–8°C.

This suspension was mixed and the cell nuclei counted in a Bürker haemocytometer, using an immunofluorescence microscope (Ortholux II, Leitz, FRG) with incident as well as white light to allow simultaneous identification of the lines in the haemocytometer and the cell nuclei. All the nuclei in eight A size squares (0.8 mm²) were counted, and the counts (C) converted to the concentration of target cells in blood by the formula:

\[
C \times \frac{VCN}{BV \times 0.8} \times 10^6 \text{ cells/l}
\]

where \( VCN \) represents the volume of the suspension of cell nuclei and \( BV \) denotes the volume of blood in the rosette suspension (Table 1).

Isolation of mononuclear cells

PBMC were obtained by Isopaque-Ficoll (Lymphoprep, Nycomed AS, Oslo, Norway) gradient centrifugation (Böyum 1968) and washed twice in Hanks balanced salt solution (Gibco Ltd, Paisley, Scotland).

Immunofluorescence

Immunofluorescence staining of PBMC was carried out using OKT11, OKT4, OKT8 and HH1 as first layer MoAb in flow cytometry studies to identify lymphocytes of the CD2, CD4, CD8 and CD37 (B cells) respectively. In immunofluorescence microscopy, the MoAb coated onto microspheres were also used as first layer MoAb. FITC conjugated goat anti mouse (GAM) IgG + IgM (Tago Inc. Burlingame, CA, USA) was used as a second layer. E2-14 (an MoAB specific for β2 microglobulin) was used as positive control and 2F4 (an MoAb with no known specificity) was used as a negative control. The cells were examined in an immunofluorescence microscope or run through a flow cytometer (Ortho cytofluorograf type 50 H, Ortho Instruments, Westwood, MA, USA). When the flow cytometer was used for the quantification of lymphocyte subsets, a window was made on the basis of forward and right-angle scatter to define a cell population which included both lymphocytes and monocytes. The percentage of target cells (TC) as a fraction of all the nucleated cells in this window was calculated using the formula:

\[
TC = \frac{M - N}{P} \times 100
\]

where \( M \) is the number of cells identified by the CD-specific MoAb, \( N \) is the number of cells identified by 2F4 (negative control) and \( P \) is the number of cells identified by E2-14 (positive control). Blood was taken at the same time for determination of the total leucocyte concentration (WBC) (Coulter Counter Ltd, Coulter Electronics Ltd, Luton, Beds, UK), and a blood smear was stained with May-Grünewald Giemsa stain and used for determination of the mononuclear cells (MNC) as a fraction of all leucocytes. This cell population was considered to correspond with that defined by the window in the flow cytometer. The total number of target cells could then be determined by the formula:

\[
\text{WBC} \times \text{MNC} \times \text{TC}
\]

Data analysis

Results from the experiments comparing lymphocyte subsets counted immunomagnetically and using the flow cytometer are presented using the Spearman rank correlation coefficient.

RESULTS

Method design

The ratio of microspheres to target cells required for the isolation of all the cells of the subset to be counted was found to be 35–50:1, using the upper limit of normal number of cells for each subset as the basis for the calculation (Table 1). The time required for the complete subset isolation was found to vary with the avidity of the MoAb. Microspheres coated with high avidity MoAb would form rosettes with all the target cells in less than 20 min, but in the context of all the subsets being counted at the same time, 1 hour was found to be optimal. The volume of
the suspension in which cell nuclei were counted (VCN) was chosen as the smallest volume where the concentration of microspheres did not interfere with the free admixture of the cell nuclei (Table 1). Blood samples could be stored at 4°C or room temperature for 24 h with no appreciable change in the lymphocyte subset counts (data not shown).

Specificity and efficiency of lymphocyte subset isolation
Positive IM selection of lymphocyte subsets directly from blood has been shown to achieve >99% pure and functionally intact cell subsets (Funderud et al., 1987). We have demonstrated specificity of target cell isolation by the fact that depletion of one lymphocyte subset did not significantly interfere with the counts of the remaining non-overlapping subsets (Fig. 1). Non-specific binding of antibody-coated microspheres to cells was tested using Dynabeads M-450-SAM (not coated with MoAb) and found to represent less than 1% of the cells counted. The efficiency of target cell subset isolation was tested by the examination of the blood left after IM isolation and quantification of a lymphocyte subset. A repeat IM quantification using microspheres coated with the same MoAb showed that more than 97% of the target cells had been isolated initially (Fig. 1). Indirect immunofluorescence assays showed that less than 2% of the mononuclear cells left in the blood after IM isolation of a lymphocyte subset carried surface markers specific for that subset (Table 2).

Comparison of IM counting and flow cytometric quantification of lymphocyte subsets
CD2+, CD4+, CD8+ and B lymphocytes were counted in blood samples from 15 donors using both the IM and the flow cytometric quantification methods. These experiments were run as a ‘blind’ comparative study by two independent investigators. The results are shown in Fig. 2 and demonstrate a significant correlation, all sixty observations giving a Spearman rank correlation coefficient of $r_s = 0.973$ $(P < 0.0002)$. However, flow cytometric quantification generally gave slightly higher figures than the IM quantification.

Reproducibility
CD2, CD4, CD8 and CD19 subsets were isolated and counted immunomagnetically five times in the same sample of blood. Results are given in Table 3. With the final suspension volumes chosen to give cell nuclei counts of 150 or more per eight A size squares (Table 1), the methodological variability was generally less than 10% from the mean of the observations. Higher cell nuclei counts tended to give less deviation from mean.

**DISCUSSION**

This paper describes a new method for the direct quantification of lymphocyte subsets in blood by means of superparamagnetic
monosized microspheres coated with MoAb specific for the various lymphocyte subsets to be counted. More than 97% of the cells of each subset were isolated and counted, the isolated cell subsets have been shown to be >99% pure, and the counting method involved a methodological variability of less than 10%. The method is fast and simple; quantification of four different lymphocyte subsets can be carried out in less than 2 h.

This method compares favourably with other methods for quantification of lymphocyte subsets. It allows the absolute enumeration of the cell subsets, in contrast to conventional techniques where absolute numbers of cells can be calculated only on the basis of a total leucocyte count, a differential counting of mononuclear cells in blood smears and the determination of the fraction of target cells in PBMC. Methodological variations may easily cause errors in such indirect, multi-step procedures, affecting perhaps most significantly small subsets of lymphocytes. This may be the main reason for the less highly significant statistical correlation between B cells counted immunomagnetically and using flow cytometry. Quantification of lymphocyte subsets by the indirect method using flow cytometry tended to give slightly higher figures than IM quantification. One reason for this discrepancy may be that the filters in the flow cytometer removed monocyte aggregates, leading to an overestimation of the lymphocyte fractions by this method. For the CD8 subset, however, the IM method tended to give slightly higher counts than the indirect method. This may reflect a selective loss of CD8+ cells during Isopaque-Ficoll separation (Renzi & Ginnns, 1987).

IM quantification has advantages also in the fields of laboratory personnel safety and laboratory economy. Because the cells are being kept in the same test tube throughout the procedure, the risk of spread of potentially infectious material is minimalized. There are no steps where aerosols are being formed, in contrast to conventional methods where both gradient centrifugation and flow cytometry may involve aerosol formation. Requirements with regard to laboratory equipment as well as technician time is reduced compared to existing methods.

The demand on immunology laboratories for lymphocyte subset quantification is likely to increase in the future along with the development of the human immunodeficiency virus (HIV)

Table 3. Methodological variability (lymphocyte subsets from the same sample of blood were isolated and counted five times on the same day)

<table>
<thead>
<tr>
<th>Lymphocyte subset</th>
<th>Test number</th>
<th>Maximal deviation from mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor A*</td>
<td>1</td>
<td>1150†</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1240</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1140</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1170</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1110</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>1160</td>
</tr>
<tr>
<td>CD2</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>CD4</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td>680</td>
<td>610</td>
</tr>
<tr>
<td></td>
<td>630</td>
<td>630</td>
</tr>
<tr>
<td>Donor B</td>
<td>270</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Donor A</td>
<td>174</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>189</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>164</td>
<td>178</td>
</tr>
<tr>
<td>CD19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Blood samples from two different donors (A and B).
† Number of cells x 10⁶/l.

Fig. 2. Comparison between IM quantification of lymphocyte subsets and indirect quantification using a total leucocyte count, a differential white cell counting and flow cytometry. ( ) CD2+, (●) CD4+, (■) CD8+ and (▲) B cells were counted in the blood from 15 donors. Spearman rank correlation coefficients (r) for all observations (n=60) and for each lymphocyte subset (n=15): All observations, r1=0.97 (P<00002); CD2+ cells, r2=0.91 (P=00003); CD4+ cells, r3=0.80 (P=00014); and B cells; r4=0.56 (P=0.018).
epidemic. Furthermore, the advent of new MoAb will expand the repertoire of lymphocyte subsets which may be enumerated. We believe the presently described method is suited to meet these challenges.

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