Clin. exp. Immunol. (1977) 29, 162–167.

Enumeration of lymphocyte populations in whole peripheral blood with alkaline phosphatase-labelled reagents

A METHOD FOR ROUTINE CLINICAL USE

M. DRUGUET* & M. B. PEPYS *Department of Immunology, Royal Free Hospital, Pond Street, London*

(*Received* 23 *December* 1976)

SUMMARY

An improved method is described for the enumeration of lymphocyte surface markers in whole peripheral blood using reagents labelled with alkaline phosphatase. A suspension of washed whole blood is exposed to the labelled reagents and then smeared on slides. Endogenous peroxidase in monocytes is detected by the diaminobenzidine reaction amplified by osmication, and this identifies more cells than are recognised as monocytes by morphological criteria in Romanovsky-stained films. Lymphocytes are identified as peroxidase-negative mononuclear cells and those binding alkaline phosphatase-labelled reagents are demonstrated by treating the smears with naphthol ASMX phosphoric acid and fast red TR salt. By avoiding the loss of lymphocytes which is inevitable in any procedure for isolation of mononuclear cells from the blood, and by permitting elimination of monocytes from the counts, this method enables the proportion and absolute number of different circulating lymphocyte populations to be accurately enumerated. In the peripheral blood of seventeen normal individuals alkaline phosphatase rabbit $F(ab)'_2$ anti-human immunoglobulin stained the following numbers (mean $\pm$s.d.) of lymphocytes, $9{\cdot}0 \pm 1{\cdot}5\%$, $214 \pm 66/\mu l$ (B cells), and specific rabbit anti-T-cell serum followed by alkaline phosphatase goat $F(ab)'_2$ anti-rabbit immunoglobulin stained $77 \pm 3\%$, $1846 \pm 488/\mu l$ (T cells). The method, which is applicable to any surface marker which can be detected on living cells in suspension with a soluble reagent, provides permanent preparations which are counted in an ordinary light microscope and permits the use of counterstaining to reveal cellular morphology. Provided that appropriate specific reagents are available it is therefore suitable for routine clinical application.

INTRODUCTION

We have recently described an immunofluorescence method for the enumeration of lymphocyte populations by the identification of surface markers on cells in whole blood (Pepys *et al.*, 1976; Pepys, 1976). This method, by avoiding procedures for separating mononuclear cells which invariably give subtotal yields and may distort the ratio between different lymphocyte populations (Brown & Greaves, 1974), permitted the accurate enumeration of both the proportion and the absolute number of specific populations in the circulation. Lymphocytes were distinguished from monocytes by their lack of intracellular peroxidase (Preud'homme & Flandrin, 1974). The whole technique is straightforward and reproducible, but it is demanding in terms of the high-quality fluorescence microscope and the time and experience required for counting. An attempt was therefore made to improve the method and make it suitable for routine clinical use. This has been achieved by employing enzyme-labelled reagents to provide permanent preparations for counting in the ordinary light microscope, and the precision and efficiency of monocyte identification have also been evaluated and improved with respect to earlier studies.

* Present address: INSERM Unité de recherches de pathophysiologie digestive, Hôpital Edouard-Herriot, Lyon, France.

Correspondence: Dr M. B. Pepys, Department of Immunology, Royal Free Hospital, Pond Street, London NW3 2QG.

MATERIALS AND METHODS

Antisera. Rabbit anti-human immunoglobulin and goat anti-rabbit immunoglobulin were raised by immunization with human and rabbit IgG respectively. The IgG antigen was isolated from normal serum by salt fractionation and DEAE–cellulose chromatography. Pure antibody from these antisera was isolated by elution from the specific IgG antigen coupled to polyacrylamide beads (Bio-Gel P300 100–200 mesh, Bio-Rad Laboratories Ltd, Kent) (Ternynck & Avrameas, 1976). Some of the pure antibody was digested with pepsin at 2% w/w to yield $F(ab)'_2$ fragments which were separated by gel filtration on Sephadex G-150 (Pharmacia (G.B.) Ltd, London). Rabbit anti-monkey thymocyte serum was extensively absorbed with normal, leukaemic and cultured human leucocytes to yield a specific anti-T-cell reagent and was provided by Dr G. Janossy and Dr M.F. Greaves (Greaves & Jarossy, 1976).

Coupling of enzymes to antibodies. Alkaline phosphatase (AP, Sigma Chemical Co. Ltd, Surrey, grade VII and grade III) was coupled to IgG and $F(ab)'_2$ antibodies with glutaraldehyde (EMscope, London) (Avrameas, 1969). The AP-coupled reagents were millipore-filtered and kept at 4°C in 2% bovine serum albumin (BSA, Sigma), 0·001 M magnesium chloride and 0·02% sodium azide. Although the present data were all obtained with AP-coupled reagents prepared by this method, there was always substantial loss of antibody activity. A two-step method, in which AP (1·0 mg) is first activated by glutaraldehyde (0·05%) and the excess coupling reagent then removed before addition of antibody (0·5 mg) is apparently much more satisfactory, particularly if the glutaraldehyde is centrifuged at 100,000 *g* for 1 hr immediately before use (W.D. Brighton, personal communication). Horseradish peroxidase (HRP, RZ = 3, Sigma) was coupled to antibody by the method of Nakane & Kawaoc, 1974).

Detection of enzymatic activity. Peroxidase activity was detected by the method of Graham & Karnovsky (1966). Smears fixed with ethanol–formaldehyde were incubated for 10 min at room temperature with a solution of 1·0 mg/ml of 3′3′-diaminobenzidine –HCl (DAB, Sigma) in 0·2 M Tris–HCl buffer, pH 7·6, containing 0·01% hydrogen peroxide. They were then washed with phosphate-buffered saline (PBS), pH 7·3. The colour reaction was amplified by incubation for 30–60 sec in 1% osmium tetroxide (Sigma) in phosphate buffer, pH 7·2. Non-specific esterase was detected in smears fixed and stained as described by Li, Lam & Yam (1973). Alkaline phosphatase was detected by incubation of the fixed smears for 1 hr at room temperature with a freshly prepared and filtered mixture of equal volumes of a solution in distilled water of 0·4 mg/ml of naphthol ASMX phosphoric acid (Sigma) and a solution in 0·2 M Tris–HCl buffer, pH 8·2, of 6 mg/ml fast red TR salt (Sigma) (Stage & Avrameas, 1976).

Whole-blood method. 250 ml of fresh venous blood anticoagulated with EDTA or heparin was washed three times (200 *g*, 10 min, 4°C) in plastic tubes (LP3, Luckham Ltd, Sussex with PBS containing 0·2% BSA and 0·2% azide (PBS–BSA–az.) 25 ml of washed packed cells were then incubated on ice for 30 min with 25 μl of an optimal dilution of specific anti-human reagent. After three washes in PBS–BSA–az smears were then prepared in the case of direct tests, whereas for indirect tests the cells were incubated again under the same conditions with an optimal dilution of an enzyme-labelled reagent. After finally washing the cells three more times, the cell pellet was reconstituted in 25 μl of undiluted foetal calf serum (Gibco Biocult Ltd, Paisley, Scotland) and thin smears were made on clean glass slides. The smears were dried for 5 min in air, fixed in a solution of 4% formaldehyde in absolute ethanol, washed with water and dried in air. The presence of AP or HRP on cells was detected by the methods described above, and in the case of slides stained for AP, the endogenous peroxidase of granulocytes and monocytes was demonstrated after the reaction for AP. After completion of the enzyme reactions the slides were washed with distilled water, dried in air and then counterstained with 1% aqueous methyl green for 2–5 min. They were then dipped briefly into water and blotted dry. Some smears from each preparation were not treated with enzyme substrate, but were stained directly with Leishmann's or Giemsa–May–Grünwald stains.

Examination of stained smears in the AP method. Smears were examined either mounted or unmounted by ordinary visible-light illumination. Monocytes were identified as mononuclear cells containing brown intracytoplasmic deposits, whereas lymphocytes were identified as mononuclear cells without any brown intracytoplasmic material. Lymphocytes stained by specific AP-labelled reagents were identified by the presence of red granular deposits. In order to minimise the effects of the heterogeneous distribution of cells within the smears each one was examined throughout its length and at least 300 total or thirty-five positive lymphocytes were counted. The absolute number of lymphocytes stained by each reagent was the product of the percentage stained and the absolute lymphocyte count. This was valid since it had been established that the washing procedures to which blood was subjected before smearing did not alter either the total or the differential leucocyte count (Pepys *et al.*, 1976).

Enumeration of circulating lymphocyte populations in normal individuals. Seventeen healthy normal subjects (ten female, seven male) aged 22–69 years (mean $\pm$ s.d. = 40·1 $\pm$ 15·1 years) were studied. Cells stained by anti-human immunoglobulin reagents were detected with both IgG and $F(ab)'_2$ antibodies directly coupled to AP. T cells were detected by indirect testing with rabbit anti-T serum and AP-goat $F(ab)'_2$ anti-rabbit immunoglobulin antibody. In all tests the reagents had been titrated and were used at their optimal concentration.

Identification of monocytes. Peripheral blood smears of five or ten individuals were stained in parallel for endogenous peroxidase, for non-specific esterase and with Giemsa–May–Grünwald, and the percentage of cells identifiable as granulocytes, monocytes and lymphocytes in each stain were counted.

RESULTS

Detection of monocytes

A comparison of the percentage of cells among peripheral leucocytes which were identified as lymphocytes by morphological criteria in Romanovsky-stained smears with the percentage identified by cytochemical criteria is shown in Tables 1 and 2. It was clear that even when the counts were conducted by a skilled haematologist more cells contained endogenous peroxidase than were recognised as monocytes by their morphology, and this difference was even greater when an observer without extensive haematological experience did the counts. In contrast non-specific esterase was mostly present in large monocytes and was absent from the small 'lymphocyte-like' cells which contained peroxidase. This use of the DAB reaction for peroxidase, amplified by osmium tetroxide, was therefore adopted as the best available marker for monocytes, with the additional advantage that it also stained granulocytes very deeply.

Selection of an enzyme for labelling antibody

In the light of the observations above it was necessary to choose an enzyme label which would be compatible with, and distinguishable from, the endogenous peroxidase in monocytes. HRP is an enzyme label with which considerable experience has been gained, for which a simple and efficient coupling

TABLE 1. Identification of peripheral blood leucocytes by morphological and cytochemical criteria

Individuals	Number of cells (% of all leucocytes)			
	Morphology		Endogenous peroxidase	
	Granulocytes + monocytes	Lymphocytes	Positive cells	Negative cells
1	76	24	81	19
2	89	11	92	8
3	67	33	72	28
4	74	26	81	19
5	70	30	72	28
6	69	31	72	28
7	82	18	88	12
8	60	40	65	33
9	89	11	92	8
10	82	18	86	14
Mean ± s.d.	75·8 ± 9·6	24·9 ± 9·6	80·1 ± 9·5	19·7 ± 9·1

TABLE 2. Frequency of mononuclear cells lacking non-specific esterase or endogenous peroxidase

Individuals	Mononuclear cells not stained (% of all leucocytes)	
	Esterase	Peroxidase
1	36	30
2	34	31
3	34	28
4	43	42
5	38	34
Mean ± s.d.	37·0 ± 3·7	33 ± 5·5

TABLE 3. Lymphocytes in whole blood stained by alkaline phosphatase-labelled reagents

Reagent	Per cent positive cells		Number of positive cells		Numbers of donors
	Mean ± s.d.	Range	Mean ± s.d.	Range	
AP-rabbit IgG anti-human immunoglobulin	13·5 ± 3·8	10·5–14·6	332 ± 120	205–351	12
AP-rabbit F(ab)′$_2$ anti-human immunoglobulin	9·0 ± 1·5	7·1–12·0	214 ± 66	133–308	17
Rabbit anti-T-cell serum + AP-goat F(ab)′$_2$ anti-rabbit immunoglobulin	77 ± 3	71–82	1846 ± 488	1056–2541	17

procedure is available and which produces a permanent colour reaction (DAB), which can be amplified by osmication. However, after treatment of cell suspensions with HRP-labelled reagents, smearing and then staining, it was not possible to distinguish the morphological appearance of surface HRP and intracellular myeloperoxidase. Attempts to develop the DAB reaction on HRP-reagent-treated cell suspensions before smearing were not successful, and the use of HRP as the enzyme label for the whole-blood method was abandoned. In contrast AP-labelled reagents were found to be suitable since the AP colour reaction using fast red was both compatible with, and distinguishable from, the DAB reaction for myeloperoxidase, and furthermore was unaffected by osmication. Best results were obtained by performing the AP reaction before the DAB reaction.

Counterstaining

Optimal results were obtained with pale nuclear counterstains contrasting in colour with the red and the brown enzyme reactions products, and methyl green was finally selected. The use of other materials than fast red for detection of AP may permit the use of counterstains which reveal better cytomorphology than does methyl green.

Use of AP-labelled reagents in the whole-blood method

The numbers of lymphocytes stained by optimal concentrations of different AP-labelled reagents in direct and indirect tests conducted at 4°C are shown in Table 3, and compared with the results in the

TABLE 4. Lymphocytes in whole blood stained by alkaline phosphatase and fluorescein labelled anti-immunoglobulin reagents

Reagent	Per cent positive cells (mean ± s.d.)	Number of donors
AP-rabbit IgG anti-human immunoglobulin	13·5 ± 2·4	12
FITC-rabbit IgG anti-human immunoglobulin	20·5 ± 1·5*	22
AP-rabbit F(ab)′$_2$ anti-human immunoglobulin	9·0 ± 1·5	17
FITC-rabbit F(ab)′$_2$ anti-human immunoglobulin	10·9 ± 3·5	11
FITC-sheep IgG anti-human IgM	8·1 ± 2·3*	22
FITC-sheep IgG anti-human IgD	8·9 ± 2·2*	11

* Data from Pepys *et al.*, 1976.

immunofluorescent technique in Table 4. As expected from earlier work (Winchester *et al.*, 1975; Pepys *et al.*, 1976), the number of cells stained by the AP-F(ab)'$_2$ anti-human immunoglobulin (9·0± 1·5%) was less than that stained by the whole-IgG reagent from the same serum (13·5±2·4%). However, the latter stained significantly less cells than IgG anti-immunoglobulin labelled with fluorescence (20·5±1·0%).

Isolated lymphocytes or whole blood washed at 4°C before immunofluorescent staining include a population of cells without stable surface immunoglobulin which adsorb IgG from the plasma; this can be eluted by washing the cells at 37°C (Lobo, Westervelt & Horwitz, 1975; Pepys *et al.*, 1976; Lobo & Horwitz, 1976). When fluorescinated IgG anti-IgG is used to stain cells prewashed at 4°C immune complexes are formed which readsorb to the cells apparently via the Fc piece of the labelled antibody, since the phenomenon is not seen when F(ab)'$_2$ anti-IgG is used (Winchester *et al.*, 1975). The present results show that labelling IgG antibody with AP interferes with this process of readsorption. Washing blood at 37°C before staining with AP-IgG anti-immunoglobulin reduced the proportion of cells stained to the same number as were stained after washing at 4°C (or 37°C) by AP-F(ab)'$_2$ anti-immunoglobulin (Table 5), which represents the B-cell population, most of which bear surface IgM and IgD (Pepys *et al.*, 1976) (Table 4).

The lymphocytes stained by the indirect method with rabbit anti-T-cell serum and AP-goat F(ab)'$_2$ anti-rabbit immunoglobulin (77%) represent the T-cell population.

As with the immunofluorescent technique the present results with the whole-blood method demonstrated considerable constancy in the proportions of the different cell populations, both in individuals and in the whole group, although the absolute numbers varied with the lymphocyte count.

TABLE 5. Effect of prewashing at 37°C on the number of lymphocytes in whole blood stained by alkaline phosphatase-labelled IgG anti-human immunoglobulin

Individuals	Per cent positive cells		
	AP-IgG anti-Ig		
	4°C	37°C	AP-F(ab)'$_2$ anti-Ig at 4°C
1	16·4	9·4	10·9
2	17·0	10·9	9·7

DISCUSSION

It is clear from the work of Brown & Greaves (1974) that for the accurate enumeration of circulating lymphocyte populations it is essential to avoid separation procedures and to study the cells in whole blood. In order to do this it is necessary to identify monocytes which share some surface markers with lymphocytes, so that they can be eliminated from the counts. In our earlier immunofluorescent version of the whole-blood technique we used endogenous myeloperoxidase as the monocyte marker, on the basis of the work of Preud'homme & Flandrin (1974). However, they found that not all monocytes identified by morphological criteria in Giemsa-stained films contained peroxidase detectable by their technique. On the contrary, in the present study it was found that the DAB method for peroxidase amplified by osmium tetroxide revealed a greater number of mononuclear cells than were identifiable as monocytes by morphological criteria alone. Non-specific esterase was detected less frequently. The adoption of peroxidase as the monocyte marker was incompatible with the use of HRP as the enzyme label for an immunoenzyme whole-blood method, and AP was therefore used.

A simple and reliable technique employing AP-labelled reagents has been devised which permits the precise and reproducible enumeration of circulating lymphocyte populations characterized by surface

markers. The present result are confined to the identification of T cells and B cells, but in preliminary work C3- and Fc-receptor-bearing populations have been demonstrated. The preparations obtained with AP-labelled reagents are observed in an ordinary light microscope and can be counted very much more easily and rapidly than the earlier immunofluorescent slides. The recognition of monocytes is simpler and there is good distinction between 'positive' and 'negative' lymphocytes. This facilitates study and contributes to accuracy. The only remaining problem is that of coupling AP without destroying antibody activity. Although satisfactory reagents can be obtained by the one-step method with glutaraldehyde, the use of a two-step technique, with prior activation of the AP, is greatly superior (W.D. Brighton, personal communication; M.B. Pepys, unpublished observations).

This immunoenzyme whole-blood method should be able to provide accurate routine clinical assessment of any lymphocyte surface marker which can be detected with soluble reagents on living cells in suspension. Furthermore, with refinement of staining technology, it will be compatible with the simultaneous demonstration of the cytomorphology of the surface-labelled cells.

Note added in proof

Since submission of this paper, the paper, the one-step glutaraldehyde method of coupling AP with antibody (Avrameas, 1966) has been found to be efficient and reproducible, with excellent preservation of antibody activity, when de-ionised water for all buffers and glutaraldehyde from TAAB Laboratories, Reading (purified for electron microscopy) are used (S. Avrameas, personal communication; M.B. Pepys, unpublished observations). Provided glutaraldehyde from this source is used, the simple one-step technique is therefore the coupling method of choice.

M. Druguet was supported by INSERM and the work was supported in part by the MRC. We wish to thank Dr Barbara Bain for her haematological expertise in performing differential leucocyte counts and Dr M.F. Greaves and Dr G. Janossy for providing anti-T-cell serum and anti-rabbit IgG.

REFERENCES

AVRAMEAS, S. (1969) Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for detection of antigens and antibodies. *Immunochemistry*, **6**, 43.

BROWN, G. & GREAVES, M.F. (1974) Enumeration of absolute numbers of T and B lymphocytes in human blood. *Scand. J. Immunol.* **3**, 161.

GRAHAM, R.C. & KARNOVSKY, M.J. (1966) The early stages of absorption of injected horse radish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**, 291.

GREAVES, M.F. & JANOSSY, G. (1976) Antisera to human T lymphocytes. *In vitro Methods in Cell-Mediated and Tumour Immunity* (ed. B.R. Bloom and J.R. David), p. 89. Academic Press, New York.

LI, C.Y., LAM, K.W. & YAM, L.T. (1973) Esterases in human leukocytes. *J. Histochem. Cytochem.* **21**, 1.

LOBO, P.I., WESTERVELT, F.B. & HORWITZ, D.A. (1975) Identification of two populations of immunoglobulin-bearing lymphocytes in man. *J. Immunol.* **114**, 116.

LOBO, P.I. & HORWITZ, D.A. (1976) An appraisal of Fc receptors on human peripheral blood B and L lymphocytes. *J. Immunol.* **117**, 939.

NAKANE, P.K. & KAWAOI, A. (1974) Peroxidase-labelled antibody. A new method of conjugation. *J. Histochem. Cytochem.* **22**, 1084.

PEPYS, M.B. (1976) Characterization and enumeration of lymphocyte populations in whole human peripheral blood *In vitro Methods in Cell-mediated and Tumour Immunity*. (ed. B.R. Bloom and J.R. David), p. 197. Academic Press, New York.

PEPYS, M.B., SATEGNA-GUIDETTI, C., MIRJAH, D.D., WANSBROUGH-JONES, M.H. & DASH, A.C. (1976) Enumeration of immunoglobulin-bearing lymphocytes in whole peripheral blood. *Clin. exp. Immunol.* **26**, 91.

PREUD'HOMME, J-L. & FLANDRIN, G. (1974) Identification by peroxidase staining of monocytes in surface immunofluorescence tests. *J. Immunol.* **113**, 1650.

STAGE, D.E. & AVRAMEAS, S. (1976) Detection of anti-TNP antibody forming cells (AFC) with TNS-enzyme and TNP-Fab anti-enzyme conjugates. *J. Immunol. Methods* **10**, 105.

TERNYNCK, T. & AVRAMEAS, S. (1976) Polymerization and immobilization of proteins using ethyl chloroformate and glutaraldehyde. *Immunoadsorbents in Protein Purification*. (ed. E. Ruoslahti), p. 29. *Scand. J. Immunol.* supplement 3, Universitetsforlaget, Oslo.

WINCHESTER, R.J., FU, S.M., HOFFMAN, T. & KUNKEL, H.G. (1975) IgG on lymphocyte surfaces: technical problems and the significance of a third cell population. *J. Immunol.* **114**, 1210.