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Identification of mononuclear cells in human blood

I. QUALTITATIVE AND QUANTITATIVE DATA ON SURFACE MARKERS AFTER FORMALDEHYDE FIXATION OF THE CELLS

H. R. E. SCHUIT,* W. HIJMANS* & G. E. M. ASMA† * *Institute for Experimental Gerontology, TNO, Rijswijk, and* † *Department of Paediatrics, University Medical Center, Leiden, The Netherlands*

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SUMMARY

The technical details of a fixation procedure with formaldehyde which was applied in a direct membrane immunofluorescence technique to mononuclear cells from normal human blood are described. After separation of the cells with Ficoll–Isopaque according to Böyum (1963) they were washed and fixed with 0·04% formaldehyde in PBS for 10 min and washed again. This cell suspension can be stored at 4°C for at least 24 hr and the slides prepared from them at −20°C for at least some months. In practice, this fixation procedure not only appeared to be effective in the preservation of cells but also showed a number of additional advantages, such as the short handling period, including the fixation procedure and the avoidance of loss of cells. Moreover, true B lymphocytes, as defined by the synthesis of immunoglobulins and the incorporation of these molecules into their cell membrane, are recognized convincingly.

INTRODUCTION

The fluorescent antibody technique is widely applied in the study of surface moieties. The large number of different cell membrane constituents and the complexity of labelled antisera, which may contain many components other than the desired specific antibody, and the various ways in which these membrane constituents and serum components may interact, either immunologically or otherwise, do not readily allow evaluation of the results observed with the fluorescence microscope. The isolation of mononuclear cells from blood according to the method of Böyum (1963) is a well established procedure; however, insufficient attention is often paid to the heterogeneity of the cell suspension which is obtained. The frequently reported high purity of this suspension is not in accordance with detailed analysis, first presented by Zucker-Franklin (1974) who mentioned the relatively large number of monocytes in these suspensions as defined by phagocytosis of latex particles in electron microscopy. Rothbarth, Hendriks-Sturkenboom & Ploem (1976) indicated that not all monocytes as defined in their histochemical technique do phagocytose latex particles. Therefore not all cells which lack this capacity should be classified as lymphocytes. Tracing with

Abbreviations used in this paper: RaHu/IgA (ab) = antibody fraction of rabbit anti-human IgA, RaHu/IgM (IgG) = IgG fraction of rabbit anti-human IgM, RaHu/IgD (IgG) = IgG fraction of rabbit anti-human IgD, RaHu/IgG (ab) = antibody fraction of rabbit anti-human IgG, RaHu/κ (IgG) = IgG fraction of rabbit anti-human kappa, RaHu/λ (IgG) = IgG fraction of rabbit anti-human lambda, RaHu/Fab (IgG) = IgG fraction of rabbit anti-human Fab, GaHu/Fab (Fab) = F(ab′)$_2$ fragment of goat anti-human Fab, HoHu/T (IgG) = IgG fraction of horse anti-human T.

Correspondence: H. R. E. Schuit, Study Group for Medical Gerontology, Department of Pathology, Leiden University Medical Centre, PO Box 9603, 2300 RC Leiden, The Netherlands.

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peroxidase according to Preud'homme & Flandrin (1974) has the disadvantage that this procedure cannot be used with live cells. Attempts to remove monocytes by iron + magnetism, or depletion of Fc receptor-bearing cells via adherence to antigen–antibody complexes, for example, also do not yield the desired results, i.e. a pure lymphocyte suspension. Moreover, any purification step involves a loss of cells and this may create confusion. In the special technical report of the WHO/IARC-sponsored workshop on human B and T cells by Aiuti *et al.* (1974) '. . .it is [therefore] recommended that no attempt be made to remove monocytes from mononuclear cell preparations to avoid selective loss of lymphocytic subpopulations'. An additional problem concerns the evidence for the presence of a separate population, called the 'third population' by Frøland & Natvig (1973) and 'L cells' by Horwtiz & Lobo (1975), Lobo, Westervelt & Horwitz (1976) and Lobo & Horwitz (1976).

Formaldehyde fixation was added to our immunofluorescence technique in an attempt to preserve cells in suspension. In practice, this fixation procedure not only appeared to be effective in the preservation of cells but also showed a number of additional advantages, which are described in this and the accompanying paper (Schuit & Hijmans, 1980).

MATERIALS AND METHODS

Handling of the cells

Preparation of the cell suspension. Coagulation of blood was prevented by 5% (w/v) EDTA (Titriplex III, Merck AG, Darmstadt, W. Germany) in phosphate-buffered saline (PBS), pH 7·2. A total leucocyte count was performed and a blood smear prepared for conventional May–Grünwald Giemsa staining.

The blood was diluted with an equal volume of PBS; 5 ml was then layered on 2 ml of Lymphoprep (Nyegaard, Oslo, Norway) and centrifuged at room temperature (22°C) for 13 min at 1,000 ***g***. The cells from the interface were suspended in PBS containing 5% bovine serum albumin (BSA; Poviet, Amsterdam, The Netherlands) and 0·1% EDTA (approximately 15 ml per interface sample), centrifuged at 4°C for 15 min at 400 ***g*** and the pellet resuspended in a 1% BSA solution in PBS to a final concentration of 2×10^7 cells·ml^{-1}.

Fixation with 0·04% formaldehyde. Commercial formaldehyde (Merck, PA, säurefrei; 37%) was diluted × 10 with PBS; this solution can be kept as a stock at 4°C for several weeks. Shortly before use, it was further diluted × 100 with PBS resulting in a final concentration of 0·04%. To 0·05 ml of the suspension containing approximately 1×10^6 cells was added 1 ml of the 0·04% formaldehyde dropwise and under gentle shaking. The mixture was kept for 10 min at room temperature. The cells were washed once with 1% BSA and the pellet was resuspended in 0·05 ml of the 1% BSA solution.

The blood should be processed as quickly as possible to prevent changes in the morphology of the cells. The period of storage should not exceed 6 hr. Titriplex III was chosen because, in our experience, it is the least toxic of the available anticoagulants. Formaldehyde fixation is a well known procedure for tissue sections. It was also applied by Smit et al. *(1974) and later by Lamelin* et al. *(1978) in the fluorescence technique for suspended cells. The concentration generally used is rather high and this appears to be toxic for cells in suspension. With 0·04% formaldehyde cell viability remains unchanged. Cap formation of lymphocytes, however, is reduced considerably. The time of fixation is rather crucial; when the cells are exposed to the diluted formaldehyde for a period longer than 10 min, morphological changes occur. The suspension of formaldehyde-fixed cells washed with the 1% BSA solution can be stored at 4°C for at least 24 hr. The addition of EDTA to the BSA washing solution prevents cell clumping.*

Staining procedure

To 0·05 ml of the cell suspension in a disposable plastic tube (70 × 11 mm), 0·025 ml of the appropriate conjugate dilution was added. Incubation was at room temperature for 30 min and the mixture was gently shaken every 10 min. The cells were washed once with 1% BSA in PBS, centrifuged in the fixed-angle Serofuge® (Clay-Adams, New York) and the supernatant removed as completely as possible. This can be achieved easily because the pellet is not located in the centre of the bottom of the tube after centrifugation in this type of centrifuge.

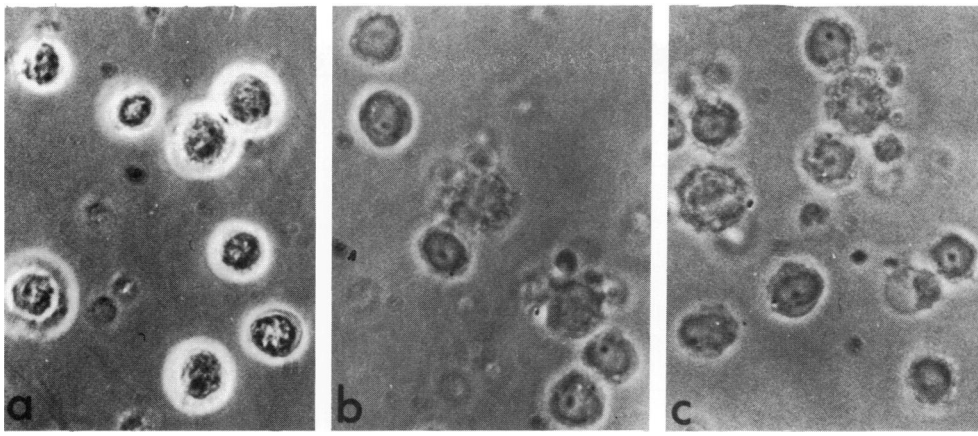

Fig. 1. Influence of embedding medium and storage on the morphology of formaldehyde-fixed mononuclear cells from human blood. Phase-contrast image of cells imbedded in (a) 1% BSA solution; (b) 90% glycerol; (c) 90% glycerol, slide kept for 6 days at −20°C. Note the absence of the halo and the fine morphological details if a high concentration of glycerol is used. (Original magnification × 1,000.)

The cell pellet was resuspended with a vortex mixer in the remaining fluid and deposited into a small drop of 90% glycerol (9 parts glycerol and 1 part PBS, pH 7·8), placed on a cover glass (24 × 32 mm). The suspension was then covered with an object glass without exerting pressure and the cover glass was sealed with paraffin.

The incubation with the conjugates at room temperature proves to be highly satisfactory. The imbedding in 90% glycerol is of paramount importance for the morphological evaluation in phase-contrast microscopy: the shape of the nucleus, the nucleolus and the details of the cytoplasm are clearly distinguishable (Fig. 1b, c). When no glycerol is used or when it is diluted too much, the morphology is markedly distorted. Also the halo interferes with a proper assessment of the morphology (Fig. 1a). Care should be taken that the glycerol stock is kept at +4°C. Paraffin has the advantage that it does not diffuse into the cell suspension, whereas nail polish based on acetone does and the acetone is toxic for cells. The slides prepared with the formaldehyde-fixed cells can be stored at −20°C for many weeks.

Antisera

For the visualization and enumeration of the T lymphocytes, a tetramethylrhodamine isothiocyanate (TRITC) labelled IgG fraction of a horse anti-human T cell antiserum was used. The preparation and the specificity of the antiserum has been described in detail by Asma, Schuit & Hijmans (1977). For B lymphocytes, a fluorescein isothiocyanate (FITC) labelled IgG fraction or an $F(ab')_2$ fraction of goat anti-human Fab was used, both obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands). These conjugates were applied to the suspension after staining with the anti-T cell antiserum. When single staining was employed, a TRITC-labelled rabbit anti-human Fab antiserum which was prepared in our Institute was used.

The combination of two reagents labelled with different fluorochromes provides a control for specificity: because all cells exposed are viewed in one slide, accurate judgement can be made concerning the number of cells which are positive for one or the other reagent and cells which have reacted with both reagents or with neither. If only a single conjugate is used, TRITC is the fluorochrome of choice for a number of reasons, the most important of which is that there is less fading. This may explain why TRITC-labelled antisera usually yield a slightly higher relative number of positive cells than do FITC-labelled antisera.

For the detection of Ig class and light chain type on the B lymphocytes, specific anti-heavy and -light chain antisera were used. The specificity was checked by performance testing, i.e. by the cytoplasmic staining of plasma cells in fixed cytocentrifuge slides prepared from the bone marrow of

Table 1. Specificity of anti-human heavy and light chain antisera. Results of performance testing on lymphoid cells of lymphatic leukaemia

	Leucocytes ($\times 10^9$/l)	RaHu/IgA* (ab)	RaHu/IgM (IgG)	RaHu/IgD (IgG)	RaHu/IgG (ab)	RaHu/κ (IgG)	RaHu/λ (IgG)	HoHu/T (IgG)
CLL	81	0·5	18	14	0·5	18	0	7
CLL	223	0	97	81	0	98	0	2
CLL	90	0	78	89	0	88	0	7
ALL	38	0	89	0·5	0	94	0·5	3
CLL	6	0	70	3	0	0	90	8
CLL	143	0	1	29	<0·5	47	0	6
CLL	22	95	<0·5	<0·5	2	90	0	7
CLL	37	0	<0·5	<0·5	78	88	0	12
CLL	38	<0·5	<0·5	<0·5	<0·5	0·5	0·5	95
ALL	195	0	<0·5	<0·5	0	0	0	1

* Figures represent the number of positive cells as a percentage of all mononuclear cells.

patients suffering from myeloma or Waldenström's macroglobulinaemia as described in detail by Hijmans, Schuit & Klein (1969) and by the membrane staining of lymphoid cells from the blood of patients suffering from lymphatic leukaemia (Table 1). The heavy chain-specific conjugates directed against IgM and IgD and the light chain-specific conjugates against kappa and lambda were prepared from IgG fractions of the antisera. The heavy chain-specific conjugates against IgA and IgG were antibody preparations isolated on immunoadsorbent columns in our Institute by Dr J. Radl.

Quantification of subpopulations

At least 200 mononuclear cells were classified according to their morphology and their reactivity. As a reference value, the expression in absolute numbers of T and B lymphocytes per ml is more informative. These values were calculated from the total leucocyte count and the differential leucocyte count.

Microscopy and photomicrography

The cells were first examined for morphology in a Leitz Dialux® microscope (Wetzlar, W. Germany) using low-voltage transmitted light and an oil-immersion phase-contrast objective lens ×63/1·30 and oculars ×6·3. The transmitted light beam was then screened by hand and each cell was examined individually for its immunological reactivity employing narrow-band excitation using a Ploem (1967) vertical illuminator containing the filter sets suitable for selective visualization of FITC and TRITC fluorescence. A mercury HBO 100-W lamp served as the light source. Photomicrography was performed on Ilford HP 4 or HP 5 film with a Leitz Orthomat®. In this automatic camera, exposure can be performed either by measuring the whole field (field measurement) or by measuring one individual cell (point measurement). For phase-contrast micrographs, field measurement was used and the cells were exposed in the FITC filter combination of the illuminator without actual excitation. Point measurement was used for the fluorescence recording in order to obtain reproducible results. The film was developed in Promicol® and prints were prepared on Agfa® Brovira Speed. The exposure time is highly dependent on the brightness of the fluorescence, but, in general, 1–2 min for FITC and 30 sec–1 min for TRITC is sufficient.

RESULTS

The fluorescent staining of the formaldehyde-fixed cells exposed to a TRITC anti-T cell conjugate followed by an FITC anti-Fab conjugate shows three different patterns. One is a clearly membrane-

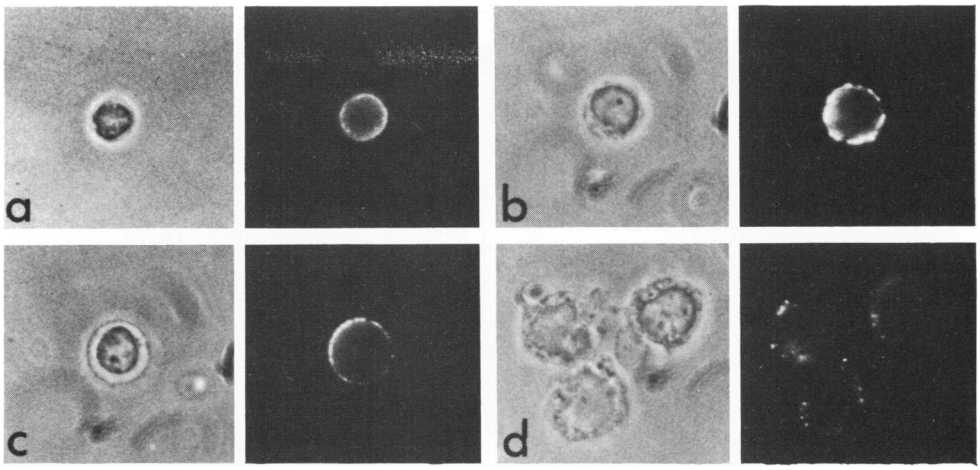

Fig. 2. Morphology and immunofluorescence microscopy of formaldehyde-fixed mononuclear cells from normal human blood. Typical membrane fluorescence of lymphocytes with (a) TRITC-HoHu/T (IgG), (b) TRITC-RaHu/Fab (IgG), (c) FITC-RaHu/IgD (IgG). Atypical membrane fluorescence of mature monocytes with (d) FITC-GaHu/Fab (Fab). (Original magnification × 1,000.)

associated confluent ring, observed on the lymphocytes which are positive with the anti-T cell reagent (Fig. 2a). The second is a clearly membrane-associated patchy fluorescence of the lymphocytes which are positive for the anti-Fab reagent (Fig. 2b) or for a heavy chain-specific conjugate (Fig. 2c). We classify these patterns as typical because they can be considered to reflect the interaction of the antiserum with components which are exclusively present in the lymphocyte membrane. The third pattern observed consists of irregularly scattered spots which are weakly stained and which are not clearly membrane-associated (Fig. 2d). This staining pattern is found in mature monocytes and called atypical because it reflects the interaction of the anti-Fab conjugate with molecules which are not exclusively restricted to the cell membrane, but which seem to have been internalized by the cell.

Table 2. Subpopulations in normal human blood. Percentage of mononuclear cells after formaldehyde fixation

Donor	T^+ (T lymphocytes)	Fab^+ (B lymphocytes)	T^+ Fab^+	T^- Fab^-	Mature monocytes†
1	61·6	4·6	0	18·7	15·1
2	50·5	4·5	0·3	19·3	25·4
3	49·3	1·4	0	26·1	23·2
4	54·5	3·5	0·3	24·3	17·4
5	64·9	2·6	0	4·8	27·7
6	67·1	2·6	0	14·8	15·5
7	64·4	8·9	0·8	4·8	21·1
8	54·0	4·0	0·2	5·8	36·0
$\bar{x}$	58·3	4·0	0·2	14·8	22·7
s.d.	7.0	2·2	0·3	8·7	7·1
$\bar{x}$	$1{\cdot}2 \times 10^6$/ml*	$0{\cdot}08 \times 10^6$/ml*			
s.d.	$0{\cdot}4 \times 10^6$	$0{\cdot}03 \times 10^6$			

Mononuclear cells tested with TRITC anti-T cell antiserum and subsequently with FITC anti-Fab antiserum.

* T and B lymphocytes expressed as absolute number/ml.

† As defined by morphology and atypical reactivity with anti-Fab conjugate.

Table 3. Subdivision of B lymphocytes in normal human blood. Percentage of mononuclear cells after formaldehyde fixation.

Donor	IgA	IgM	IgM+IgD	IgD	IgG	κ	λ
1	4·8	0·4	2·7	0·4	0	1·8	2·0
2	2·2	0·9	1·4	0	1·5	2·5	1·0
3	1·5	0	0·5	0	0·5	0·5	0·5
4	4·0	0·4	3·9	2·2	0·4	1·4	2·0
6	2·0	0	4·0	0	0·5	3·0	1·5
9	3·0	0	4·2	2·1	0·5	3·7	5·0
10	4·1	0	7·0	2·6	1·0	3·4	6·5
11	3·4	0	7·8	0·9	0	3·8	2·8
$\bar{x}$	3·1	0·2	4·2	1·0	0·5	2·5	2·7
s.d.	1·1	0·3	2·6	1·1	0·5	1·2	2·1

Mononuclear cells tested with either TRITC-labelled anti-IgA and -IgG (antibody preparations) conjugates, a combination of FITC anti-IgM and TRITC anti-IgD (IgG fractions) conjugates, or with a combination of FITC anti-κ and TRITC anti-λ (IgG fractions) conjugates.

This phenomenon can be observed clearly when the cells are not fixed as shown in Fig. 1e in the accompanying paper (Schuit & Hijmans, 1980). It is less pronounced after formaldehyde fixation (Fig. 2d).

The cells which react with the anti-T cell antiserum are mainly small lymphocytes with a small rim of cytoplasm and a nucleus showing one distinct nucleolus. Approximately 25% of the T-positive lymphocytes are larger lymphocytes. The cells reacting in a typical way with the anti-Fab conjugate show a similar morphology as the T lymphocytes, but the number of larger B lymphocytes is very small. Mononuclear cells reacting with both T and B cell reagents are occasionally seen; the mean relative number is 0·2%. About 15% of the total number of mononuclear cells is negative for both reagents. The percentage of lymphocytes which give a typical pattern with anti-Fab reagents is about equal to the percentage of light chain-positive lymphocytes, but it is smaller than the sum of the relative numbers of heavy chain-positive lymphocytes. The mature monocytes have a morphology clearly different from the lymphocytes; the cell is much larger and has abundant cytoplasm and a polymorphous nucleus. As mentioned above, they show a weak atypical fluorescence with the anti-Fab conjugate, which is not clearly membrane-associated. With these two characteristics, mature monocytes can be easily distinguished. They also stain with the same weak atypical pattern for IgG and for both light chains simultaneously. The $F(ab')_2$ fraction of a goat anti-Fab antiserum gave the same atypical fluorescence as did the IgG fraction. Quantitative results are given in Tables 2 and 3.

DISCUSSION

Application of a method in which formaldehyde fixation, embedding in 90% glycerol, phase-contrast and double-wavelength fluorescence microscopy are combined, greatly facilitates the evaluation of the morphology and the immunological reactivity with antisera of the mononuclear cells from normal human blood. Both morphological and immunological criteria are then used for the classification of an individual cell. The classification of the cells which are positive with the anti-T cell antiserum does not present a problem. Morphologically, these cells can be defined as small or larger lymphocytes. The cells which are positive with the anti-Fab conjugate, however, do not belong to one subpopulation of mononuclear cells. The relative number of cells showing typical (i.e.

clearly membrane-associated) fluorescence with the anti-Fab conjugate is approximately equal to the relative number of cells which are positive for both IgM and IgD and of which the staining pattern is also typical. It may be concluded therefore that these cells are true B lymphocytes. The fact that the relative number of typical Fab-positive lymphocytes is smaller than the sum of the relative numbers of heavy chain-positive lymphocytes could indicate the presence of multiple heavy chains on individual B lymphocytes as observed by Gathings, Lawton & Cooper (1977) in neonatal B lymphocytes and our own findings (Vessière-Louveaux, Hijmans & Schuit, 1980a, b) in adult human blood and tonsils.

It is difficult to explain why an occasional cell exhibits reactivity with both the anti-T cell antiserum and the anti-Fab conjugate. The obvious conclusion that a few lymphocytes may have both T and B lymphocyte features should not be drawn too easily. Perhaps this double specificity may be attributed to autoantibodies directed against a small proportion of T lymphocytes. The second subpopulation of mononuclear cells which is positive with the anti-Fab conjugate consists of mature monocytes. About 25% of the mononuclear cells obtained by the Ficoll–Isopaque separation technique are mature monocytes. These cells are easily distinguishable because of their morphology in phase-contrast microscopy combined with their atypical fluorescence with the anti-Fab reagent. The presence of IgG molecules on formaldehyde-fixed mature monocytes was demonstrated. Because of the fact that both light chains are present, it is very unlikely that these molecules are synthesized by the cell itself; these findings rather suggest a specific interaction of the antiserum with autologous cell-bound IgG molecules.

Evidence was found for a separate population of mononuclear cells which failed to show fluorescence with anti-immunoglobulin antisera, apart from the typical B lymphocytes with their typical fluorescence pattern and the monocytes with their atypical fluorescence. To investigate the possibility that the so called 'third population' or 'L cells' detected by Frøland & Natvig (1973), Horwitz & Lobo (1975) and Lobo *et al.* (1976) belong to the population which is completely negative after formaldehyde fixation, experiments which are reported in the accompanying paper (Schuit & Hijmans, 1980) were performed.

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