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Identification of mononuclear cells in human blood

II. EVALUATION OF MORPHOLOGICAL AND IMMUNOLOGICAL ASPECTS OF NATIVE AND FORMALDEHYDE-FIXED CELL POPULATIONS

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SUMMARY

The presence of surface-associated immunoglobulins and Fc receptors on mononuclear cells from normal human blood was investigated by the direct immunofluorescence technique combined with phase-contrast microscopy. Formaldehyde-fixed cells were compared to unfixed cells and to cells preincubated at 37°C. In the unfixed samples a separate population which showed Fc receptors in an immunofluorescence technique using a labelled antigen–antibody complex was detected. This cell population showed an atypical, i.e. not clearly membrane-associated, pattern of fluorescence with anti-Fab conjugates. This interaction most probably is due to autologous IgG molecules taken up by these cells from the donor serum. Using phase-contrast microscopy, these cells were morphologically distinct from lymphocytes and mature monocytes. They will be referred to as 'undefined mononuclear cells' (UMC). After formaldehyde fixation or preincubation at 37°C the interaction of the UMC with anti-Fab conjugates could no longer be demonstrated. Mature monocytes show the same atypical fluorescence pattern with anti-Fab conjugates, but in contrast to the UMC the interaction persists after formaldehyde fixation or preincubation at 37°C. No evidence was found for passive uptake of labelled IgG from conjugates by any mononuclear cell. $F(ab')_2$ fragments of IgG from antisera gave results similar to those obtained with intact IgG fractions. The morphology of the different cell subpopulations is described and their relative numbers in normal blood are given. Formaldehyde fixation proved to be a simple and useful procedure, especially for the determination of the number of B lymphocytes, because the Fc receptor of the undefined mononuclear cells does not give rise to confusion.

INTRODUCTION

Frøland & Natvig (1973) and Frøland, Natvig & Michaelsen (1974), Horwitz & Lobo (1975) and Lobo, Westervelt & Horwitz (1976) have presented evidence for the existence of a separate population of mononuclear cells in human blood bearing Fc receptors and membrane-labile IgG. From the data of Lobo & Horwitz (1976) and Kumagai *et al.* (1975) it appeared that these IgG molecules are released by preincubation of the cells at 37°C in a serum-free medium. These cells were initially considered to have the features of a lymphocyte. Electron microscopy studies by Horwitz *et al.* (1978), however, suggested the possibility that these cells are neither lymphocytes nor mature monocytes. In our studies (Schuit, Hijmans & Asma, 1980) with formaldehyde-fixed mononuclear

Abbreviations used in this paper: RaHu/Fab (IgG) = IgG fraction of rabbit anti-human Fab, GaHu/Fab (Fab) = $F(ab')_2$ fragment of goat anti-human Fab.

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cells, a subpopulation was distinguished which failed to react with anti-Fab conjugates or with an anti-T cell conjugate.

To obtain more information on this subpopulation and in addition on the interaction of the Fc receptor in the immunofluorescence technique for the detection of surface immunoglobulins, these studies have now been extended. A labelled antigen–antibody complex was used for the detection of Fc receptors and the reactivity with different anti-immunoglobulin conjugates was compared in formaldehyde-fixed cells, in unfixed cells and in cells preincubated at 37°C.

MATERIALS AND METHODS

The preparation and the handling of the cells has been described in the preceding paper (Schuit *et al.*, 1980). Samples contained approximately 1×10^6 cells in 0·05 ml of a 1% BSA solution in PBS. Either the samples were processed without fixation or fixed with 0·04% formaldehyde, or incubated in 1 ml of RPMI 1640 containing 10% foetal calf serum for 1 hr at 37°C. Tubes were kept in a vertical position. After centrifugation in a serofuge, the cell pellet was washed with 3 ml of 1% BSA at 37°C and resuspended in 0·05 ml of this solution.

Antisera. In the experiments in which unfixed cells, formaldehyde-fixed cells and cells preincubated at 37°C were compared, a tetramethylrhodamine isothiocyanate (TRITC) labelled rabbit anti-human Fab antiserum was used along with a mixture of two TRITC-labelled rabbit antisera, one directed against human IgM and the other against human IgD; this mixture of antisera recognizes almost all B lymphocytes; Vessière-Louveaux, Hijmans & Schuit (1980) give a figure of 96%. The number of UMC and mature monocytes was determined with a fluorescein isothiocyanate (FITC) labelled goat anti-human Fab conjugate.

The origins of the antisera used in the experiments in which $F(ab')_2$ fragments of IgG from antisera were compared with intact IgG fractions are given in Table 4. To investigate the occurrence of Fc receptors for IgG on B lymphocytes, the mixture of the two TRITC-labelled antisera directed against IgM and IgD was used. For the T lymphocytes, this was performed with a TRITC-labelled IgG fraction of a horse anti-human T cell antiserum described by Asma, Schuit & Hijmans (1977).

Detection of Fc receptor. The presence of an Fc receptor for IgG was investigated by the direct immunofluorescence technique using a labelled antigen–antibody complex; the method described by Winchester & Ross (1976) was slightly modified by using phosphate-buffered saline (PBS), pH 8·3, for the dilution of the antiserum and for the antigen solution. The complexes were prepared fresh and added to the cell suspension. A rabbit anti-ovalbumin antiserum was inactivated for 30 min at 56°C and diluted 1/4. Of this diluted antiserum, 0·1 ml was added to 0·025 ml of a 0·2 $mg \cdot ml^{-1}$ FITC-labelled ovalbumin. The mixture was kept at room temperature for 30 min and 0·025 ml of the supernatant was added to 0·1 ml of the cell suspension containing approximately 1×10^6 cells. The cells were then washed with 1% BSA in PBS and embedded in 90% glycerol as described in detail in the preceding paper (Schuit *et al.*, 1980).

Quantification of cell subpopulations. At least 200 mononuclear cells were examined. The numbers in the samples which were pretreated at 37°C could not be compared to the untreated or the formaldehyde-fixed samples because of the loss of mature monocytes (see Results section). In these experiments, these cells therefore were excluded from the counting.

RESULTS

Formaldehyde fixation caused a significant decrease in the number of cells which reacted with anti-Fab conjugates. No decrease was observed in the number of cells reactive with the anti-IgM + IgD antiserum (Table 1). The cells which stained with anti-Fab conjugates when unfixed and which became negative after formaldehyde fixation could be distinguished as a separate population in both morphological and immunological aspects. They will be referred to as undefined mononuclear cells (UMC). From the morphological point of view, these cells differ from classical lymphocytes: although their nucleus is round or oval, the cell border is not distinct, the cytoplasm is less

Table 1. Comparison of formaldehyde-fixed mononuclear cells, unfixed and 37°C pretreated cells

	Unfixed			1 hr at 37°C		Formaldehyde--fixed	
Donor	Fab+	μδ+	Fc+*	Fab+	μδ+	Fab+	μδ+
16	20	5	n.d.	8	2	13	9
17	34	16	23	18	13	26	16
18	11	3	5	2	3	3	2
19	11	4	5	2	3	5	3
20	20	15	3	15	11	14	11
21	19	14	3	16	11	14	11
$\bar{x}$	19	9	8	10	7	12	9
s.d.	8	6	8	7	5	8	5

* As defined by the binding of antigen–antibody complex.
n.d. = Not done.

Mononuclear cells tested with a TRITC-labelled rabbit anti-Fab or a mixture of anti-IgM + IgD conjugates or with a complex of rabbit anti-ovalbumin + FITC-labelled ovalbumin.

Figures represent the number of positive cells as a percentage of mononuclear cells with the exclusion of mature monocytes.

dense than that seen in a lymphocyte of the same size and often vacuoles can be observed. From the immunological point of view, the fluorescence of the UMC with an anti-Fab conjugate is atypical, i.e. not clearly membrane-associated (Fig. 1d) and comparable to the atypical fluorescence of the mature monocytes (Fig. 1e), whereas the B lymphocyte shows a typical membrane-associated fluorescence (Fig. 1a, b, c). After formaldehyde fixation, the atypical fluorescence of the UMC is no longer detectable, but the atypical staining pattern of mature monocytes persists. The results obtained after incubation at 37°C are similar with respect to the reactivity with the anti-Fab reagent: disappearance of the atypical fluorescence of the UMC, but not of the mature monocytes. During incubation at 37°C, many mature monocytes are lost because of their adherence to plastic,

Table 2. Effect of reincubation with autologous or homologous plasma on unfixed mononuclear cells pretreated at 37°C

	1 hr at 37°C		1 hr at 37°C, 30 min at 4°C (autologous plasma)		1 hr at 37°C, 30 min at 4°C (homologous plasma)	
Donor	Fab+	μδ+	Fab+	μδ+	Fab+	μδ+
16	8	2	13	5	n.d.	n.d.
17	18	13	39	10	n.d.	n.d.
19	2	3	8	1	11	n.d.
20	15	11	21	11	23	7
21	16	11	16	11	24	11

n.d. = not done.

Mononuclear cells tested with a TRITC-labelled rabbit anti-Fab or a mixture of anti-IgM + IgD conjugates.

Figures represent the number of positive cells as a percentage of mononuclear cells with the exclusion of mature monocytes.

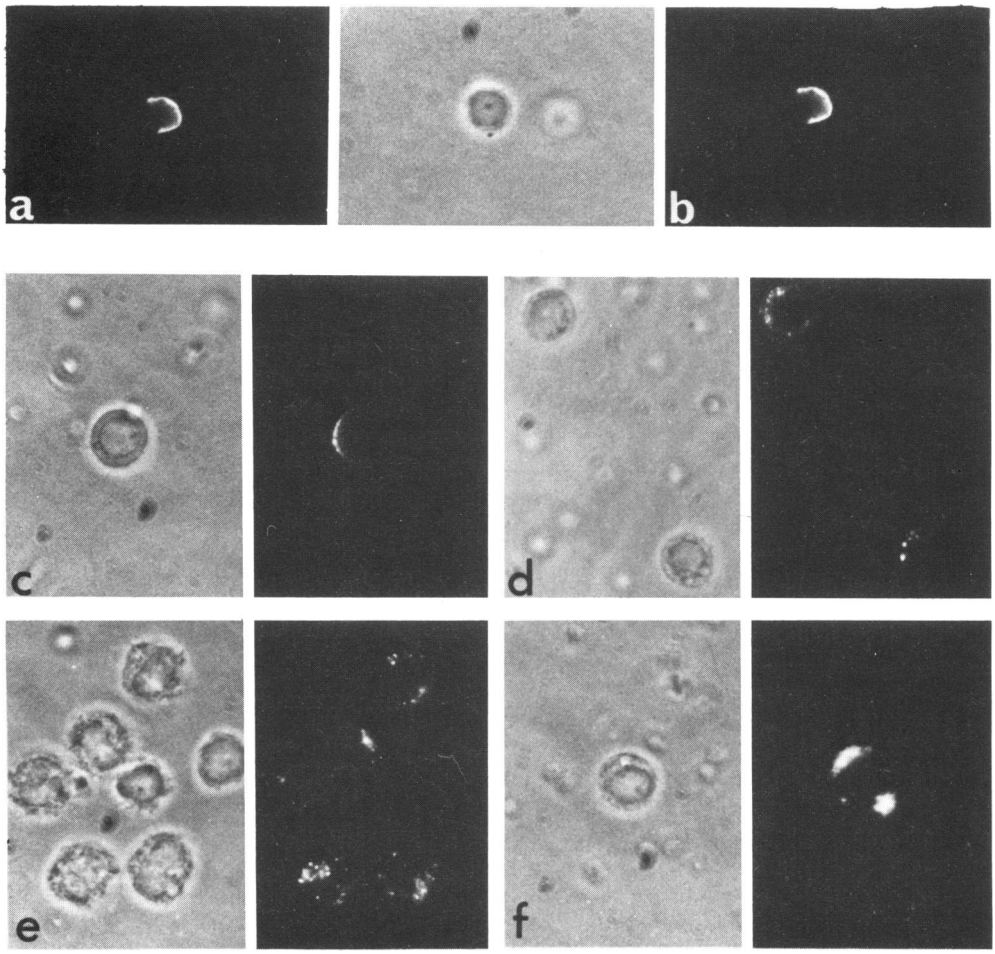

Fig. 1. Morphology and immunofluorescence microscopy of unfixed mononuclear cells from normal blood. Typical membrane fluorescence of a small lymphocyte. The same cell is shown in phase contrast and after staining with (a) TRITC-RaHu/Fab (IgG), (b) FITC-GaHu/Fab (Fab). Typical membrane fluorescence of (c) larger lymphocyte with TRITC-RaHu/Fab (IgG). Atypical membrane fluorescence of (d) undefined mononuclear cells and (e) monocytes with TRITC-RaHu/Fab (IgG), where the cells show internalization of the molecules; (f) binding of antigen–antibody complex by UMC. In one block the same cells have been photographed in phase contrast and in immunofluorescence. (Original magnification × 1,000.)

but the suspension is not completely depleted of monocytes. The UMC seem not to be adherent, because, after reincubation with autologous or isologous plasma, the number of Fab-positive cells is approximately equal to the number found in the unfixed sample (Tables 1 and 2). It is likely that a small percentage of the B lymphocytes is no longer recognized after the incubation at 37°C for 1 hr (Table 1), probably because of shedding of the Ig molecules. Neither the UMC nor the mature monocytes show fluorescence with an anti-IgG conjugate specific for the heavy chain when unfixed, whereas unfixed B lymphocytes containing membrane-associated IgG molecules are positive. The staining of both the undefined cells and the monocytes with anti-light chain conjugates is weak and variable when fixation is not applied, but, if detectable, both light chains are present simultaneously. In the fixed preparations, both light chains and γ chains could be detected on all monocytes. As mentioned above, the UMC are completely negative after formaldehyde treatment.

The majority of the cells which bind antigen-antibody complexes exhibit the same morphology as the undefined cells (Fig. 1f) and the average number of these cells is comparable to the number of UMC (Table 3). Formaldehyde fixation causes a decrease in the number of cells which bind the antigen–antibody complex from an average of 11·8 to 6·4% ($n=4$). The majority of the UMC could

Table 3. Subpopulations in normal human blood showing atypical fluorescence with anti-Fab or binding of antigen–antibody complex

	Reactivity with:		
	Anti-Fab		
Donor	Mature Monocytes	Undefined Cells	Antigen–antibody complex
1	18·0	15·2	14·6
2	32·0	17·0	13·0
3	41·7	11·0	7·6
4	23·0	9·5	12·0
5	27·7	11·0	10·3
6	17·9	14·0	7·0
7	20·9	7·2	3·5
8	33·5	8·0	8·1
$\bar{x}$	27·1	11·6	9·5
s.d.	8·7	3·5	3·6

Mononuclear cells tested with FITC-labelled goat anti-Fab conjugate.

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

Table 4. Comparison of IgG and F(ab′)$_2$ fractions of antisera on unfixed or formaldehyde-fixed mononuclear cells of normal human blood

	Unfixed		Formaldehyde-fixed	
Antiserum	IgG fraction	F(ab′)$_2$ fraction	IgG fraction	F(ab′)$_2$ fraction
Anti-IgA	1·0 ± 0·4 (3)	2·3 ± 1·3 (3)	1·9 ± 1·8 (8)	1·0 ± 0·9 (3)
Anti-IgG	0·4 ± 0·2 (4)	0·6 ± 0·9 (4)	0·7 ± 0·9 (9)	2·3 ± 1·1 (4)
Anti-IgM	3·8 ± 2·9 (4)	4·7 ± 4·3 (4)	4·3 ± 2·1 (11)	3·8 ± 1·8 (8)
Anti-IgD	n.d.	n.d.	4·8 ± 3·1 (11)	3·3 ± 1·0 (4)
Anti-Fab	13·5 ± 4·3 (13)	12·9 ± 5·2 (13)	4·0 ± 1·9 (10)	4·6 ± 1·9 (14)

Figures represent the number of positive cells as a percentage of total mononuclear cells followed by the number of tests in parenthesis.

Antisera were raised in the following laboratories from the respective animals indicated:

(a) *IgG fraction.* Nordic Immunological Laboratories, Tilburg, The Netherlands: anti-IgA, anti-IgG (swine); anti-Fab (goat). Dr J. Radl and P. van den Berg, Institute for Experimental Gerontology, Rijswijk: anti-IgM, anti-IgD (rabbit).

(b) *F(ab′)$_2$ fraction.* Dr. F. Skvaril, Institute for Clinical and Experimental Cancer Research, University of Berne, Switzerland: anti-IgA, anti-IgG (sheep). Dr W. Knapp, Institute for Immunology, University of Vienna, Austria: anti-IgM, anti-IgD (rabbit)—both fractions prepared from the same antiserum as the IgG fractions. Nordic Immunological Laboratories, Tilburg, The Netherlands: anti-Fab (goat).

Table 5. Morphological and immunological properties of mononuclear cells in Ficoll–Isopaque-separated normal human blood

	Morphology	Reactivity in immunofluorescence microscopy
Small lymphocyte	Small cell, round nucleus, always one clear nucleolus, very thin rim of cytoplasm, distinct cell border	*Typical*, strong, clearly membrane-associated Either for T or for Fab In general: $\mu+\delta$; occasionally: α or γ Either κ or λ Only occasional binding of ag–ab complex
Larger lymphocyte	Larger cell, round nucleus, larger rim of cytoplasm which has a dense appearance, distinct cell border	
Undefined mononuclear cell	Larger cell, nucleus round or oval, ample cytoplasm, which has a thin appearance and often vacuoles, cell border not distinct	*Atypical*, weak, not clearly membrane-associated For Fab, occasionally $\kappa+\lambda$ After formalin fixation completely negative, majority binds ag–ab complex
Mature monocyte	Larger cell, polymorphous nucleus, abundant cytoplasm, cell border not distinct, irregular	*Atypical*, weak, not clearly membrane-associated For Fab, occasionally $\kappa+\lambda$ After formalin fixation positive for Fab, γ, $\kappa+\lambda$ Minority binds ag–ab complex

not be identified as T or B lymphocytes when FITC-labelled antigen–antibody complexes were applied in combination with either the TRITC-labelled anti-T cell antiserum or the mixture of TRITC-labelled antisera directed against IgM and IgD: 9·5% of the cells binding the complexes were positive with the anti-T cell antiserum and 0·4% were B lymphocytes. Conversely, T or B lymphocytes only occasionally showed binding of the complexes: 1·2% of the T lymphocytes and 4% of the B lymphocytes.

$F(ab')_2$ fragments of IgG did not show a significant difference when compared to the IgG fractions of the antisera (Table 4). Particularly, the $F(ab')_2$ fragments of IgG of a goat anti-Fab conjugate gave the same atypical staining pattern in UMC and mature monocytes as did the unsplit IgG fraction. In addition, the atypical fluorescence was always independent of the animal source of the anti-Fab antiserum, i.e. goat antiserum reacted in the same way as rabbit antiserum. The morphological and immunological properties of the subpopulations detectable in normal human blood are summarized in Table 5.

DISCUSSION

Five different populations of mononuclear cells could be distinguished in Ficoll–Isopaque-separated normal human blood when the two-wavelength immunofluorescence method was combined with phase-contrast microscopy after staining with a TRITC-labelled anti-T cell antiserum and a FITC-labelled anti-Fab conjugate. Approximately 4% of the cells could be classified as B lymphocytes, 60% as T lymphocytes, 25% as mature monocytes and approximately 1% as null cells. The classification of the remaining 10% still presents a problem. These latter, as yet undefined cells (UMC), can bind antigen–antibody complexes and are therefore assumed to have a receptor for the Fc part of IgG. According to Forni & Pernis (1975) this method is the most efficient for this purpose. Combination with phase-contrast microscopy allows a more accurate judgement of the

morphology of the cells showing Fc receptors than in the rosette technique, in which IgG-coated erythrocytes are used. The fluorescence procedure also offers the possibility of demonstrating the simultaneous occurrence of Fc receptors and surface immunoglobulin or surface T antigen. Because all cells exposed are viewed in one slide, double specificity or non-reactivity can be investigated with accuracy. The application of heat-aggregated IgG was not successful in our hands. The UMC differ morphologically from classical lymphocytes as well as from mature monocytes (for details, see Results section and Table 5). They differ from B lymphocytes in that the fluorescence pattern with an anti-Fab conjugate is not clearly membrane-associated. In this respect, they resemble mature monocytes but, in contrast to these cells, the atypical staining pattern of the undefined cells is abolished by formaldehyde fixation.

The atypical reaction of these latter two subpopulations with an anti-Fab conjugate is most likely due to the presence of autologous IgG on the cell membrane, presumably bound to an Fc receptor in such a way that the Fab part is still available for binding to the conjugates. In the unfixed cell samples, only B lymphocytes among the mononuclear cells show fluorescence with heavy chain-specific antisera and, in particular, no interaction of the UMC or the mature monocytes is seen with a heavy chain-specific anti-IgG conjugate. From this observation it may be concluded that unfixed mononuclear cells do not passively take up labelled IgG from conjugates. After formaldehyde fixation, IgG molecules are demonstrable exclusively in mature monocytes. Apparently, the IgG molecules taken up from the serum are made more accessible by the fixation procedure. The fact that the mature monocyte is able to endocytose may be the explanation why these cells do not show release of the IgG after incubation at 37°C, whereas the undefined cells do, probably because they are not capable of endocytosis.

We have to conclude that no receptors for the Fc part of IgG are present on the majority of T and B lymphocytes in blood in the conditions of our experiments. The absence of Fc receptors on B lymphocytes was also reported recently by Alexander & Herkart (1978).

The problems which occur in the identification of B lymphocytes in general are often attributed to the interaction of the conjugate with an assumed Fc receptor (Winchester *et al.*, 1975). As mentioned above, it seems, however, that the majority of the B lymphocytes do not possess a receptor for the Fc part of IgG. Moreover, IgG which is bound to an unfixed Fc receptor-bearing cell cannot be demonstrated by antisera specific for the heavy chains of IgG. Some of the results in the literature which are suggestive of the existence of an Fc receptor on B lymphocytes can be explained by the fact that polyvalent anti-Ig conjugates are often used for the detection of B lymphocytes. These conjugates contain antibodies to the Fab part of the Ig molecules which will react with the IgG on unfixed mature monocytes and UMC. Therefore, it should be stressed that an unfixed mononuclear cell which shows fluorescence with a polyvalent anti-Ig conjugate does not necessarily represent a B lymphocyte. Also conjugates which contain antigen–antibody complexes due to absorption with soluble antigens to render them specific can give problems. The use of this procedure is often unknown to the consumer if the conjugates are obtained commercially. The binding of these labelled complexes by the UMC will lead to their identification as B lymphocytes.

The $F(ab')_2$ fragments of IgG from antisera were compared with intact IgG fractions because Winchester *et al.* (1975) advocated the use of these fragments to avoid uptake of labelled IgG aggregates or complexes from the conjugate via the Fc receptor. However, the application of $F(ab')_2$ fragments of IgG from antisera will not help to solve these problems; if such a conjugate contains anti-Fab activity, unfixed mature monocytes and UMC are stained equally well as with the IgG fraction. When this activity is absent, i.e. when the conjugate is specific for the Fc part of the Ig molecule, these two subpopulations are not stained, regardless of whether the conjugate has been prepared by using the complete IgG or the $F(ab')_2$ fragments of the IgG from the antiserum.

Reports which suggest improved results when $F(ab')_2$ fragments are used can perhaps be explained by the fact that, after the splitting of the IgG molecules, the antiserum is passed through a Sephadex 200 column with the intent of removing intact IgG molecules. Antigen–antibody complexes can also be removed by this procedure.

For the correct characterization and enumeration of B lymphocytes in unfixed samples, neither the anti-Fab nor the anti-polyvalent Ig antisera are reliable reagents; antisera specific for the Fc part of the Ig molecules are to be preferred. As stated in the previous paper,

fixation with diluted formaldehyde, however, offers an attractive alternative, because the UMC are modified by the fixation procedure in such a way that they do not interact with the applied anti-Fab conjugate and mature monocytes can be easily identified because of their morphology combined with a clearly atypical fluorescence pattern.

The undefined cells are most likely identical to the cells designated as 'third population' by Frøland & Natvig (1973) and 'L cells' by Horwitz & Lobo (1975). Because they lack the specific membrane properties of T and B lymphocytes and resemble mature monocytes in certain aspects, the question arises as to whether they are immature monocytes.

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