Detection of small numbers of immature cells in the blood of healthy subjects

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Abstract

Aims—To determine the frequency of immature haemopoietic cells in the peripheral blood of healthy persons.

Methods—Cytocentrifuge preparations were made using mononuclear leucocytes separated by a Ficoll-Hypaque density gradient. The slides were stained by May-Grünwald-Giemsa. The combination with immunoperoxidase technique allowed immunotyping of uncommon blood cells.

Results—Blast cells expressing the progenitor cell marker CD34 represented 0.11 (0.06) per cent (mean (SD)) of the total mononuclear leucocyte count; these were the haemopoietic progenitor cells in the peripheral blood. Dark blue cells expressing CD38, CD45, HLA-DR, CD4, CD11a, CD29, CD49d, CD50, and CD54 represented 0.30 (0.21) per cent of the mononuclear leucocytes; most of these cells did not express T, B, NK, myelomonocytic, progenitor cell, proliferation, activation, blood dendritic cell, or follicular dendritic cell markers. These were dendritic cell precursors in the peripheral blood. Very small numbers of cells expressing CD83 were found. Blast-like cells expressing

CD45, HLA-DR, CD11a, and CD50 represented 0.15 (0.10) per cent of the mononuclear leucocytes; morphology and immunotyping supported the conclusion that these cells were poorly differentiated monocytes.

Conclusions—Morphological investigation of mononuclear leucocytes in peripheral blood of healthy persons can be used to detect small numbers of blasts, dark blue cells, and blast-like cells. The immunoperoxidase technique can then be used for immunotyping of these cells. This simple method may be helpful in diagnosing haematological disorders.

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Microscopy of blood smears yields much information about the blood cells. However, a definitive diagnosis of haematological disease is not possible in all patients. We used a simple method to improve the results of blood examination. Mononuclear leucocytes were separated using a Ficoll-Hypaque density gradient and cytocentrifuge preparations were made. The slides were stained by May-Grünwald-Giemsa. When combined with the immunoperoxidase technique,¹ this allowed us to immunotype uncommon blood cells. The analysis of these uncommon normal blood cells is the basis for identifying abnormal cells in haematological disease. Further studies will analyse the value of this technique in haematological disorders.

Table 1 Monoclonal antibodies used in the study

Antibody	CD	Expression	Source
WM 35	1a	Langerhans cells	Cymbus Biotechnology
MT910	2	T cells	Dako
Leu4	3	T cells	Becton-Dickinson
13B8.2	4	T helper cells	Coulter-Immunotech
Leu1	5	T cells	Becton-Dickinson
OKT8	8	T suppressor cells	Ortho
253.1	11a	LFA	Coulter-Immunotech
Mo1	11b	MAC	Coulter-Immunotech
BU15	11c	p 150/95	Coulter-Immunotech
My4	14	Monocytes	Coulter-Immunotech
7E4	18	Integrin β 2	Coulter-Immunotech
B-Ly1	20	B cells	Dako
4KB128	22	B cells	Dako
2A3	25	Interleukin-2-receptor	Becton-Dickinson
Lia 1/2	29	Integrin β 1	Coulter-Immunotech
HPCA-1	34	Progenitor cells	Becton-Dickinson
T16	38	Plasma cells	Coulter-Immunotech
T29/33	45	Common leukocyte antigen	Dako
Gi9	49b	VLA-a-2	Coulter-Immunotech
Hp2.1	49d	VLA-a-4	Coulter-Immunotech
HP2/19	50	ICAM 3	Coulter-Immunotech
84H10	54	ICAM 1	Coulter-Immunotech
Leu19	56	NK cells	Becton-Dickinson
Leu7	57	NK cells	Becton-Dickinson
10.1	64	Monocytes	Serotec
PG-M1	68	Monocytes	Dako
HB15	83	Dendritic cells	Coulter-Immunotech, Dr.Tedder
BT1	102	ICAM 2	Coulter-Immunotech
MPO-7		Myeloperoxidase	Dako
8a4		IgG	Coulter-Immunotech
6E2C1		IgA	Dako
R1/69		IgM	Dako
C4		λ light chains	Coulter-Immunotech
6E1		κ light chains	Coulter-Immunotech
R4/23		Follicular dendritic cells	Dako
Ki67		Proliferating cells	Dako
L243		HLA-DR	Becton-Dickinson

Table 2 Detection of uncommon cells in peripheral blood of healthy persons

	Blasts	Blast-like cells	Dark blue cells	Myelocytes and metamyelocytes
1	0.10	0.30	0.25	0.05
2	0.17	0.01	0.23	0.12
3	0.13	0.03	0.59	0.38
4	0.10	0.10	0.17	0.07
5	0.17	0.06	0.08	0.42
6	0.17	0.08	0.33	0.25
7	0.04	0.15	0.41	0.05
8	0.01	0.28	0.10	0.05
9	0.08	0.25	0.31	0.16
10	0.07	0.13	0.86	0.59
11	0.02	0.13	0.17	0.06
12	0.24	0.28	0.25	0.04
13	0.15	0.25	0.23	0.21
14	0.09	0.08	0.44	0.11
15	0.11	0.05	0.11	0.08
Mean	0.11	0.15	0.30	0.18
SD	0.06	0.10	0.21	0.17

The values are expressed as per cent of mononuclear leukocytes.

Figure 1 A blast (arrowed) in peripheral blood (May-Grünwald-Giemsa stain).

Methods

Venous blood from healthy adult donors (3 ml in EDTA) was separated by Ficoll-Hypaque density gradient centrifugation (Nycomed Pharma AS; density 1.077 g/ml) for 20 minutes at 400 *g*. The mononuclear cells were removed, washed in phosphate buffered saline (PBS), and suspended in PBS with 10% bovine serum albumin and 10% fetal calf serum (S-PBS). The suspension was then cytocentrifuged using a Shandon centrifuge. We analysed 30 000 mononuclear leucocytes on each cytocentrifuge preparation.

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The cells were investigated using a morphological-immunological technique.¹ The cytocentrifuge preparations were stained by May-Grünwald-Giemsa (five minutes with May-Grünwald, Merck No 1.01424; 20 minutes with Giemsa, Merck No 9204, diluted 1:10 with phosphate buffer, pH 7.2) and photographed (Zeiss-Axioskop). Immunocytochemical labelling was done on the same preparations after the staining, by an indirect immunoperoxidase technique in coverplates (Shandon).

The characteristics and sources of the antibodies used in this study are listed in table 1. The mouse primary monoclonal antibodies were diluted with S-PBS, with 10% human AB serum at optimal concentrations, and incubation was performed for 60 minutes. AB serum was not added during immunoglobulin analysis. Destaining of the smears was observed after this procedure.

The preparations were washed with S-PBS, and then rabbit antimouse immunoglobulin antiserum was added (peroxidase conjugated; Dako code No P260; diluted 1:50 with S-PBS/10% AB serum). After incubation for 30 minutes and washing, swine antirabbit immunoglobulin antiserum (peroxidase conjugated; Dako code No P217; diluted 1:50 with S-PBS/10% AB serum) was added for a further 30 minutes of incubation. After washing, peroxidase substrate (50 mg of diaminobenzidine hydrochloride; 10 µl of H₂O₂; 100 ml PBS) was

added for five minutes of incubation, following by counterstaining with Hämalaun (Merck No 9249) for five minutes.

Endogenous peroxidase activity was observed in eosinophils but not in other cells. Eosinophils were morphologically easy to recognise. The immunostained cells were compared with May-Grünwald-Giemsa stained cells which were photographed (see fig 2, A and B below). It was possible to observe the morphological and immunostained cytocentrifuge preparations simultaneously using a slide projector.

Specificity controls included omission of the primary antibody and use of an irrelevant primary antibody of the same isotype. We determined that the immunocytochemical reaction gave positive reactions only with cells that expressed the antigen corresponding to the primary antibody (though not in the case of eosinophils). The percentage of positive cells was compared using detection by the immunoperoxidase method after May-Grünwald-Giemsa staining and acetone fixation.

Immunoperoxidase staining of mature dendritic blood cells with CD83 was performed on cytocentrifuge preparations after fixation with May-Grünwald for three minutes. This fixation method allowed the preservation of cellular detail.

Results

We investigated the mononuclear leucocytes of 15 healthy persons. Most of the cells were lymphocytes and monocytes, but microscopic examination also showed uncommon leucocytes that are rarely encountered when performing a conventional differential leucocyte count on 100 white blood cells (table 2).

We found that 0.11 (0.06) per cent (mean (SD)) of the mononuclear leucocytes were blasts (fig 1). These circular cells (12–18 µm in diameter) have a round nucleus with only a scanty amount of pale blue cytoplasm and one or two nucleoli. The nuclear chromatin is finely dispersed and shows little clumping. May-Grünwald-Giemsa/immunoperoxidase staining showed expression of CD34 of all blasts. CD34 positivity was not detected on other mononuclear leucocytes.

We found that 0.18 (0.17) per cent of the mononuclear leucocytes were neutrophilic myelocytes and metamyelocytes, while 0.30 (0.21) percent were dark blue cells (fig 2). These latter cells (13–30 µm in diameter) have a dark blue basophilic cytoplasm with a perinuclear zone and an angulated eccentric nucleus with coarse chromatin. Cells with several nuclear lobes may occasionally be seen. These cells are morphologically easy to differentiate from plasma cells which have a circular nucleus. Immunotyping of these cells by the May-Grünwald-Giemsa/immunoperoxidase technique showed expression of CD38, CD45, and HLA-DR (table 3). Most of the dark blue cells were CD4 positive and failed to express T (CD2, CD3, CD5, CD8), B (CD20, CD22, IgG, IgA, IgM, λ and κ light chains), NK (CD56, CD57), and myelomonocytic (myeloperoxidase, CD14, CD64, CD68) cell markers.

Figure 2 Dark blue cells (arrowed) in peripheral blood. (A) May-Grünwald-Giemsa stain; (B) immunoperoxidase staining of the same cells with anti-HLA-DR; (C) May-Grünwald-Giemsa stain.

Only a small part of the dark blue cells was positive for CD2 and CD3. The dark blue cells did not show positivity for the progenitor cell marker CD34, the proliferation marker Ki67, the activation marker CD25, the marker for blood dendritic cells CD83, or the marker for follicular dendritic reticulum cells R4/23. The dark blue cells expressed the cell adhesion molecules CD11a, CD29, CD49d, CD50, and

Table 3 Investigation of antigen expression by uncommon mononuclear blood cells using the May-Grünwald-Giemsaimmunoperoxidase technique

Antigen	Dark blue cells	Blast-like cells
T cells		
CD1A	–	ND
CD2	Most—; few +	–
CD3	Most—; few +	–
CD4	Most +	–
CD5	–	–
CD8	–	–
B cells		
CD20	–	–
CD22	–	–
IgG	–	–
IgA	–	–
IgM	–	–
λ	–	–
κ	–	–
Granulocyte/monocyte		
CD14	–	–
CD64	–	Most—; few +
CD68	–	–
Myeloperoxidase	–	–
NK cells		
CD56	–	–
CD57	–	–
Progenitor cells		
CD34	–	–
Proliferating cells		
Ki67	–	–
Dendritic blood cells		
CD83	–	–
Follicular dendritic cells		
R4/23	–	–
Adhesion		
CD54	+	–
CD102	–	–
CD50	+	+
CD11a	+	+
CD11b	–	–
CD11c	–	–
CD18	Half + ; half –	ND
CD29	+	ND
CD49b	–	ND
CD49d	+	ND
Activation		
CD25	–	–
Leucocytes		
CD45	+	+
CD38	+	–
MHC		
HLA-DR	+	+

ND, not determined.

CD54. A part of the dark blue cells was CD18 positive. CD11b, CD11c, CD49b, and CD102 were not detected on the dark blue cells.

Small numbers of cells expressing CD83 (1–3/60 000 mononuclear leucocytes) were found in peripheral blood. Not all CD83 positive cells had the characteristic dendritic morphology, as shown in fig 3.

We found that 0.15 (0.10) per cent of the mononuclear leucocytes were blast-like cells (fig 4). These cells (13–20 µm diameter) are circular and have an ovoid nucleus with fine stranded chromatin and a distinct nucleolus with pale blue cytoplasm around the nucleus. Immunotyping with the May-Grünwald-Giemsa/immunoperoxidase technique showed positivity for HLA-DR, CD45, CD11a, and CD50. CD64 expression was present in a few blast-like cells. These cells failed to express T (CD2, CD3, CD4, CD5, CD8), B (CD20, CD22, IgG, IgA, IgM, λ and κ light chain), NK (CD56, CD57), myelomonocytic (CD14, CD68, myeloperoxidase), progenitor cell (CD34), dendritic blood cell (CD83), or follicular dendritic reticulum cell (R4/23) markers. The adhesion molecules CD11b, CD11c, CD54, CD102 were not found.

Figure 3 Immunoperoxidase staining of a blood dendritic cell (CD83; after three minutes of May-Grünwald fixation).

Figure 4 A blast-like cell (arrowed) in peripheral blood (May-Grünwald-Giemsa stain).

Discussion

Conventional microscopic blood smear examination of healthy persons shows polymorphonuclear leucocytes, lymphocytes, monocytes, and a few bands, eosinophils, and basophils. We investigated the mononuclear leucocytes after Ficoll-Hypaque density separation and were able to identify less common cells. Immunocytochemical investigation of these cells after May-Grünwald-Giemsa staining¹ allowed immunotyping of these cells after morphological identification. We found a small number of blasts in the peripheral blood. All these cells expressed CD34 and no other cells were CD34 positive. The use of monoclonal antibodies in conjunction with an activated cell sorter has shown that CD34 positive bone marrow cells include most progenitor cells,^{2,3} and most of these cells were found to be blast cells. Flow cytometric analysis enables CD34 positive cells to be detected in the peripheral blood and shows that there is a correlation between CD34 positive cells and granulocyte-macrophage colony forming cells.^{4,5} We conclude that the blasts which we found in the peripheral blood represent haemopoietic progenitor cells. Our

values (0.11 (0.06) per cent of mononuclear leucocytes) are in agreement with those determined by flow cytometry.⁴

We found a small percentage of dark blue cells in peripheral blood. These cells express HLA-DR, CD45, CD38, CD4, and same adhesins. Most of these cells do not stain with typical T, B, NK, and myelomonocytic markers and lack CD34, CD25, CD83, Ki67, and R4/23. These morphological and immunotyping characteristics are the same as those found in cells described as dendritic cell precursors in peripheral blood.⁶ These antigen presenting cells have a weak stimulatory capacity in an allogenic mixed leucocyte reaction (MLC) and mature in culture in dendritic cells to become potent stimulators of the MLC. The dendritic cell precursors are CD83-negative and acquire CD83 positivity as they mature.⁷ The dark blue cells have a different morphology and immunotype from plasma cells, which show positivity for light chains and IgG or IgA. Plasma cells do not express CD45 and HLA-DR.⁸ We did not find plasma cells in the peripheral blood of our healthy subjects. A small subpopulation of the dark blue cells express the T cell markers CD2 and CD3.

Dendritic cells in blood, interdigitating dendritic cells in lymph nodes, and Langerhans cells of the skin have been reported to express the CD83 antigen.⁹ It has been reported that CD34 progenitor cells which are cultured in the presence of granulocyte/macrophage colony stimulating factor and tumour necrosis factor α develop into dendritic Langerhans cells.¹⁰ It is difficult to identify cells with dendritic cell morphology in the blood.^{6,11} However, a small number of CD83+ HLA-DR+ cells without T, B, NK, and monocytic cell marker expression could be detected without purification procedures using three colour cytofluorometry.¹¹ We were able to detect a small number of cells expressing CD83 in the peripheral blood of our healthy subjects. Only a proportion of these cells had the characteristic dendritic morphology.

We also detected a small population of cells with blast-like morphology. In contrast to blasts, these cells have an ovoid nucleus with fine stranded chromatin structure and more cytoplasm. Immunotyping did not show expression of CD34, CD38, T, B, NK, or myelomonocytic markers. These cells have HLA-DR and CD45 positivity. HLA-DR positivity was found on progenitor cells, B cells, and monocytes/macrophages.¹² The blast-like cells did not show expression of CD34 for progenitor cells or CD20, CD22, and light chains for B cells. We therefore conclude that the blast-like cells are poorly differentiated monocytes. It is not surprising that these cells do not express the monocyte marker CD14 and CD68 because these antigens are not detectable on poorly differentiated monocytes.¹³ The monocyte marker CD64 is positive on a few of these cells. The morphology of the blast-like cells is an intermediate stage between blasts and monocytes. The nucleolus signifies blasts and the fine stranded chromatin of the nucleus signifies monocytes.

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- 1 Oertel J, Oertel B, Schleicher J, et al. Immunotyping of blasts in human bone marrow. *Ann Hematol* 1996;72:125-9.
- 2 Civin CI, Strauss LC, Brovall C, et al. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol* 1984;133:157-65.
- 3 Strauss LC, Rowley SD, La Russa VF, et al. Antigenic analysis of hematopoiesis. V. Characterization of My-10-antigen by normal lymphohematopoietic progenitor cells. *Exp Hematol* 1986;14:878-86.
- 4 Serke S, Säuberlich S, Abe Y, et al. Analysis of CD34-positive hemopoietic progenitor cells from normal adult peripheral blood: flow-cytometrical studies and in vitro colony (CFU-GM, BFU-E) assays. *Ann Hematol* 1991;62:45-53.
- 5 Siena S, Bregni M, Brando B, et al. Flow cytometry for clinical estimation of circulating hematopoietic progenitors for autologous transplantation in cancer patients. *Blood* 1991;77:400-9.
- 6 O'Doherty U, Steinman M, Peng M, et al. Dendritic cells freshly isolated from human blood express CD4 and mature into immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J Exp Med* 1993;178:1067-78.
- 7 Weisman D, Li Y, Anaworachich J, et al. Three populations of cells with dendritic morphology exist in peripheral blood only one which is infectable with human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* 1995;92:826-30.
- 8 Harada H, Kawano MM, Huang N, et al. Phenotypic difference of normal plasma cells from mature myeloma cells. *Blood* 1993;81:2658-63.
- 9 Zhou LJ, Tedder TF. Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J Immunol* 1995;154:3821-35.
- 10 Caux D, Dezutter-Dambuyant C, Schmitt D, et al. GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature* 1992;360:258-61.
- 11 Thomas R, Davis LS, Lipsky PE. Isolation and characterization of human peripheral blood dendritic cells. *J Immunol* 1993;150:821-34.
- 12 Schlossman S, Chess L, Humphreys R, et al. Distribution of Ia-like molecules on the surface of normal and leukemic cells. *Proc Natl Acad Sci USA* 1976;73:1288-92.
- 13 San Miquel JF, Gonzalez M, Canizo MC, et al. Surface marker analysis in acute myeloid leukaemia and correlation with FAB classification. *Br J Haematol* 1986;64:547-60.