Haematology and Blood Transfusion Vol. 28
Modern Trends in Human Leukemia V
Edited by Neth, Gallo, Greaves, Moore, Winkler

Antigen Expression on Normal and Leukaemic Erythroid Precursors

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A. Introduction

Erythroleukaemia is rare, comprising no more than 5% of acute myeloid leukaemias. This low incidence is curious, since the involvement of different cell lineages in leukaemia is likely to reflect progenitor cells "at risk" of transformation, and myeloid progenitors do not occur with a greater frequency than those of the erythroid lineage. One possible explanation is that myeloid progenitors are more "sensitive" target cells for leukaemogenesis than erythroid progenitors. However, clonal analysis has shown that although acute myeloid leukaemia can involve a target cell with differentiation restricted to the granulocyte macrophage lineage, it may frequently involve a pluripotent stem cell target [5, 11, 20], and therefore the limited maturation observed is preferentially non-erythroid.

An alternative explanation, suggested by Andersson [1], is that erythroleukaemia is in fact more common than we realise, but that it is incorrectly diagnosed as poorly differentiated acute myeloid (M1-FAB classification) [4] or even acute lymphoblastic leukaemia. Using a rabbit antiserum specific for the majored cell membrane protein glycophorin A, he found that 15% of M1 AML and 10% of relapsed ALL were glycophorin A positive. Some cases were also positive with an antibody to fetal haemoglobin.

We have used a series of monoclonal antibodies, including two specific for glycophorin A, together with fluorescence-activated cell sorting and clonal cultures of haemopoietic progenitors to determine the pattern of antigen expression during normal erythropoiesis, and have compared this with the pattern observed in acute leukaemias.

B. Methods

Normal bone marrow was obtained by aspiration from adult volunteers, centrifuged on Ficoll-isopaque, and the interface mononuclear cells collected, washed and suspended in Eagles medium +2% fetal calf serum. $20-100 \times 10^6$ bone marrow cells were incubated for 30 min at 4 °C with previously determined optimal concentrations of monoclonal antibody (see below), washed and then stained with an affinity purified $F(ab')_2$ preparation of goat anti-mouse antibodies which had been cross absorbed with insolubilized human immunoglobulin and labelled with fluorescein isothiocyanate (FITC). The cells were washed twice and then analysed and sorted on a modified fluorescence activated cell sorter (FACS-1, Becton Dickinson). Cells were processed in sterile conditions using relative fluorescence intensity to separate positive from negative cells. Cytospin preparations were made from aliquots of the unfractionated control and from each fraction, stained with May-Grünwald-Giemsa, and differential leucocyte counts performed.

Culture Procedures. Unfractionated controls and positive and negative fractions were cultured for erythroid colonies in methyl cellulose [12] in the presence of 2–2.5 u erythropoietin (Connaught Step III); erythroid colony forming units (CFU-

E) were counted at 7 days and burst-forming units (BFU-E) at 14 days.

Granulocyte-macrophage colonies (CFU-GM) were cultured in a mixture containing 25% FCS, 1% BSA, antibiotics, 0.9% methylcellulose and 5% PHA-leucocyte conditioned medium and CFU-GM counted on day 13 or 14. Recovery per 10^5 unfractionated cells was determined for each fraction and expressed as a percentage of the total colony recovery.

A similar analysis was carried out for precursors using the cytospin differential counts.

Leukaemic samples (heparinized blood and bone marrow) were obtained from referring hospitals throughout the United Kingdom as part of a routine immunodiagnostic service. All samples were separated on Ficoll-isopaque and binding of murine monoclonal antibodies assessed with FITC $F(ab')_2$ goat anti-mouse IgG using both fluorescence microscopy (Standard 16 Zeiss photomicroscope with epi-illuminescence) and flow cytometry (FACS-I).

For intranuclear TdT staining cytospin slides were fixed in cold methanol, incubated with rabbit antibodies specific for TdT [6], followed, after washing, by fluorochrome-labelled goat antibodies specific for rabbit IgG. The E rosette test was performed by standard methods using neuraminidase-treated sheep red blood cells.

C. Antibodies

I. Mouse Monoclonal Antibodies

LICR.LON.R.10 and LICR.LON.R.18 were produced as previously described [2, 10]. Ascitic IgG was used and titred by indirect immunofluorescence using erythrocytes, the cell line K562 [13] and the FACS. The dilution used was twice the minimal concentration giving saturated (maximal) staining intensity.

Other monoclonal antibodies used include J-5 anti common-ALL [17]; OKT1, OKT3, OKT6, OKT9, OKT10, and OKT11a [14, 16]; DA-2 anti HLA-DR and W6/32 anti HLA-ABC [8] and AN51 anti-platelet glycoprotein I [15]; OKM1 anti-monocyte/granulocyte [7]; R6A-anti band 3 [2]; and MAS 016 [3] and N16 [9] anti-blood group A.

II. Human Monoclonal Antibodies

Human Monoclonal antibodies to blood group I (Stephenson) and i (Dench) were obtained from Dr. K. Shumach (Toronto General Hospital).

III. Rabbit Anti-Human Transferrin

(DAKO).

D. Changes in Cell Surface Antigen Expression During Haemopoietic Differentiation

The patterns of antigen expression during normal haemopoiesis have been established using antibodies defining blood group (A, I/i), HLA-associated (-ABC and -DR), lineage-specific and transferrin receptor antigens. Details of these results are published elsewhere [18, 19] and summarized in Fig. 1.

Like HLA-DR and ABC the antigen defined by OKT10 is expressed on the earliest progenitors and lost during differentiation, suggesting a possible role in interactions regulating the proliferation of these cells.

In contrast, blood group A antigen is strongly expressed on only a small proportion of early erythroid and myeloid progenitors but increases during differentiation. I antigen shows a similar increase in expression during erythropoiesis but little expression on myeloid cells. Conversely, i antigen shows little expression on erythroid progenitors but is expressed on erythroid precursors and on myeloid progenitors and precursors.

Erythroid lineage specific antigens glycophorin A (gA) and band 3 are not present on erythroid progenitors and first expressed on maturing normoblasts. Similar results for gA expression have now been obtained using the monoclonal antibody R.10 and a rabbit anti-gA kindly provided by Dr. L. Andersson. There is no significant expression of these determinants on myeloid cells.

Transferrin receptors, defined by an antibody to transferrin itself or by the monoclonal OKT9, are expressed on most BFU-E and virtually all CFU-E but only a smal-

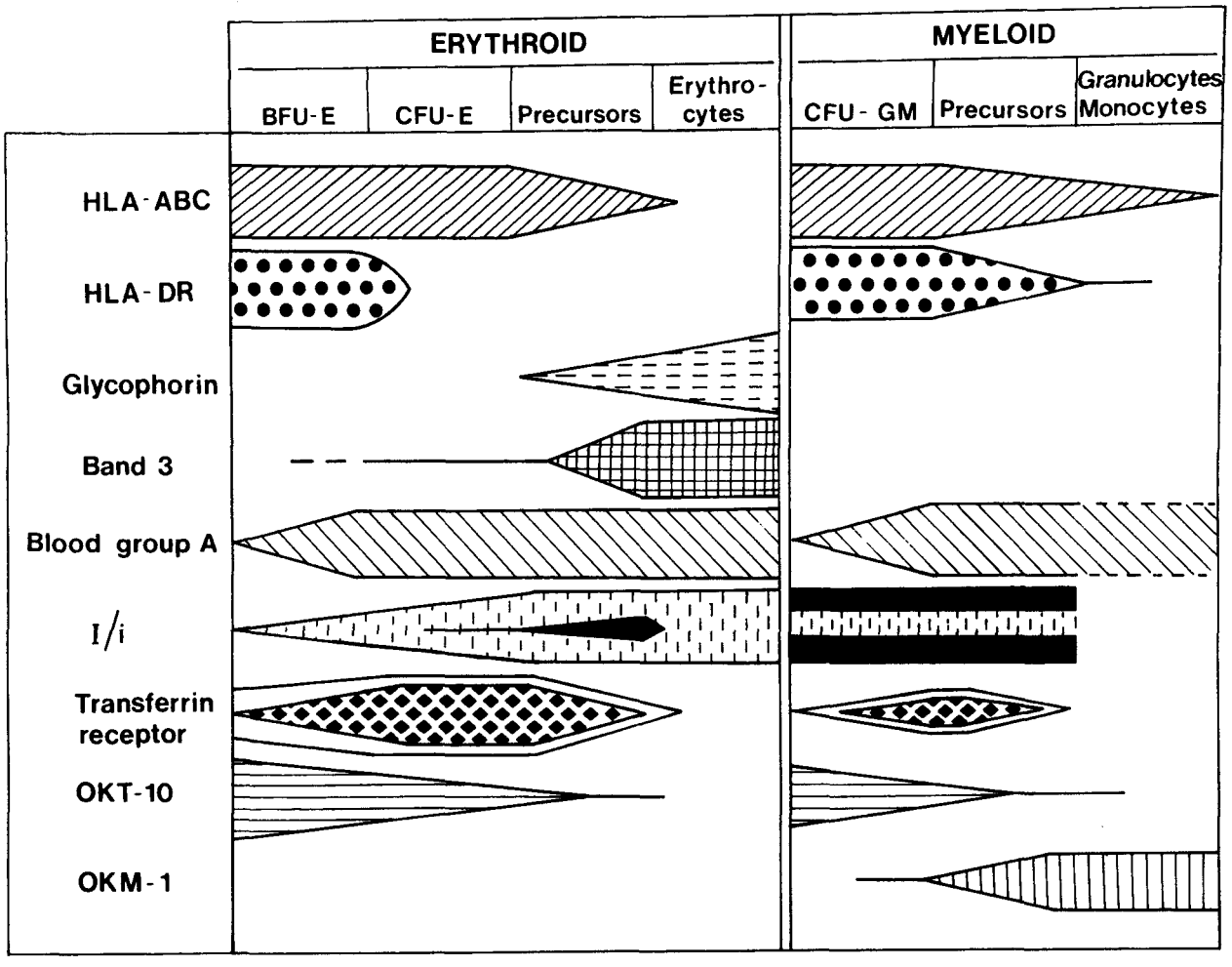

Fig. 1. Summary of monoclonal antibody defined cell surface phenotype of erythroid and myeloid cells. *Width of bars* approximates to relative proportion of antigen-positive cells in various maturation compartments. Blood group I/i: *solid, black area* represents i expression. Transferrin receptor: *hatched area* represented expression of monoclonal OKT9 defined determinant compared with the transferrin-binding site (*total area*)

ler proportion of myeloid cells and therefore may be useful for selecting and enriching for erythroid progenitors. They are not, however, specific for cells of the erythroid lineage.

E. Monoclonal Anti-glycophorin as a Probe for Erythroleukaemia

Since it is evident from the above data that we do not have an antibody specific for erythroid progenitors, it is clear that for the purpose of comparison with leukaemic phenotypes these data are incomplete; erythroleukaemias originating in either pluripotent stem cells or early erythroid progenitors, and those with maturation 'arrest' at these stages of differentiation, would not be expected to be gA+, unless asynchromy of gene expression had occurred. Furthermore, they would not be diagnosable as erythroid unless they could be induced to differentiate to gA+ or haemoglobin synthesizing cells in vitro. Seven hundred and fifty one cases of leukaemia, either at presentation or relapse, were assessed for glycophorin positivity. A variable proportion of gA+ cells were present in 27 erythroleukaemias studied; six with a high proportion of blasts had less than 15% gA+ cells while 21 had between 16% and 66% positive cells.

The overwhelming majority of non-erythroleukaemias were glycophorin A negative (Table 1), i.e. less than 10% positive cells, and this included 224 patients with acute lymphoblastic leukaemia at presentation and 103 in relapse. Four cases out of 81 common ALL in relapse had a marked increase of gA+ cells (up to 65%), but these patients were in early relapse

	Reactive	Unreactive	
		Presentation	Relapse
Erythroleukaemia	27		
Acute lymphoblastic leukaemia	2[a]		
Common ALL		149	81 (4E)[b]
T-ALL		32	10
Null-ALL		41	12
B-ALL		2	0
Acute undifferentiated leukaemia		23	4
Acute myeloid leukaemia	2+5E[b]	152	15
Acute promyelocytic leukaemia		9	0
Acute myelomonocytic/monocytic leukaemia	1	28	2
Acute megakaryoblastic leukaemia	2	3	2
Chronic myeloid leukaemia (CML)		14	0
CML blast crisis			
Erythroid	3		
Lymphoid	0	0	33
Myeloid	4	0	68
Megakaryoblastic	1		
Other		15	9

Table 1. Reactivity of different leukaemias with monoclonal anti-glycophorin

[a] Both baby girls less than 6 months old with equivocal diagnosis of ALL (see text)
[b] Clearly defined erythroid component separate from blast cells

with only a moderate increase of lymphoblasts and significant residual erythropoiesis. Separation of the gA+ cells in one case using the FACS identified greater than 85% of these cells as normoblasts. Two patients, both girls less than 6 months old, were diagnosed and treated as ALL but had a high proportion of gA+ blasts (Table 2). There was no immunological evidence to support the diagnosis of ALL (Table 2) and review of the morphology and cytochemistry showed pleomorphic blasts with basophilic-vacuolated cytoplasm and prominent granular PAS positivity. Neither patient responded to standard induction treatment for ALL, and more intensive therapy failed in the one patient who received it; both patients died without remitting. It is likely that both of these patients were in fact 'cryptic' erythroleukaemias.

There were only 18 gA+ cases out of 371 non-lymphocytic, non-erythroid leukaemias studied and none of 24 other cases including B-CLL, disseminated lymphomas, myeloma and hairy cell leukaemia (one case). Eight patients of 214 with acute myeloid leukaemia had between 15% and 50% gA+ cells; however, an obvious erythroid component was present in five of these cases. Two patients of seven with acute megakaryoblastic leukaemia were gA+; one of these patients had two cell populations demonstrable by fluorescence mi-

Table 2. Antigenic characteristics of leukemic blasts in two cases of putative 'cryptic' erythroleukemia diagnosed as ALL

Cell markers	Patient	
	D.H.	S.H.
Monoclonal antibodies		
J-5 (anti-cALL/gp100)	–	–
DA-2 (anti-HLA-DR)	–	9%
OKT1, 3, 4, 6, 8, 11A (anti-T)	–	16% – 19%
AN51 (anti-platelet glycoprotein I)	–	n.t.
OKM-1 (anti-granulocyte/monocyte)	–	–
R6A (anti-band III)	7%	n.t.
LICR.LON.R10 (anti-glycophorin A)	85%	81%
R.18 (anti-glycophorin A)	86%	n.t.
Other markers		
Sheep (E) rosettes	–	8.5%
Cell surface Ig	–	7%
TdT	–	–

– = < 5% positive cells
n.t. = not tested

croscopy and FACS analysis, large blast cells positive with the antiplatelet monoclonal AN51 and smaller gA+ blasts. This leukaemia appears to involve a progenitor common for the erythroid and platelet lineages. Eight Ph' positive blast crises of chronic myeloid leukaemia (of 109 tested) were gA+. Three of these were, however, 'erythroid' blasts crises, four myeloid and one possibly megakaryoblastic, but was negative with the platelet glycoprotein I monoclonal AN51. These data therefore differ from those of Andersson et al. [1], the number of gA+ leukaemias being considerably less than indicated in the smaller series from Finland. Part of the explanation may lie in differences in diagnostic criteria. Of the small number of AMLs that had gA+ cells in our series, most had a morphologically identifiable erythroid component, and the two interesting gA+ paediatric cases were considered on haematological grounds to be possible, though not unequivocal ALLs. Immunological data indicated that they were not ALL. Another possible explanation for the discrepancy could be differences in the antibodies used to detect gA, but this is unlikely since we found similar intensities and pattern of staining in comparative studies with rabbit anti-gA (kindly provided by L. Andersson).

We conclude from our data that the highly selective expression of gA in normal haemopoiesis is maintained in leukaemia. Glycophorin-positive leukaemias may be overt erythroleukaemias (M6 AML) or mixed leukaemias with erythroblasts and megakaryoblasts, suggesting clonal origin from a bi- or pluripotent stem cell (megakaryoblastic leukaemias and Ph' blast crisis of CML).

Only very rarely do cases diagnosed as AML or ALL express glycophorin A, and we suggest than these cases are genuine erythroleukaemias and not myeloblasts or lymphoblasts aberrantly expressing gA.

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