Changes in Surface-Membrane Components during the Differentiation of Rabbit Erythroid Cells

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The membrane components of rabbit bone-marrow-bound erythroid cells were characterized and compared with those of circulating rabbit erythroid cells. By the criteria of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, radioiodination with lactoperoxidase and binding of radioiodinated lectins, the two circulating forms of erythroid cells (the reticulocyte and erythrocyte) have the same surface components. In contrast, bone-marrow-bound nucleated erythroid cells have a unique set of membrane surface components which are completely different from those found on circulating cells. Of the ten Coomassie-Blue-staining proteins present in nucleated erythroid-cell plasma-membrane preparations, eight are accessible at the extracellular surface, and all of these are lectin-binding glycoproteins. Bone-marrow erythroid cells separated according to age by velocity sedimentation were also studied. The changeover in surface components occurs after the last nucleated stage of the erythroid cells (the orthochromatic normoblast). We discuss the alterations in membrane surface components observed during the differentiation of the erythroid-cell series in relation to the transition from bone-marrow-bound to circulating forms of these cells. We suggest that the change in membrane surface components may be linked to the loss of the nucleus from the normoblast and the entry of the erythroid cell into the circulation.

Although the structure and functions of membrane glycoproteins and cell-surface carbohydrates have long been the subjects of intensive study (Oseroff *et al.*, 1973), little is known of their role in cellular processes. The maturation of bone-marrow erythroid cells to produce free circulating erythrocytes provides an opportunity for studying changes in cell surfaces which may be directly linked to, or involved in, the control of well-described changes in cellular function. The terminal cell in the erythroid-maturation process, the erythrocyte, provides a well-known model membrane (Steck, 1974; Singer, 1974), and much is now known about the membrane proteins, carbohydrate and surface properties of this cell.

A particularly crucial stage in the differentiation of the erythroid-cell series is the change from a marrow-bound 'tissue-like' cell to a freely circulating form. Some of the changes to be expected during this event probably involve alterations in the surface properties and therefore in the membrane components of the cell.

In animals recovering from phenylhydrazine-induced anaemia the bone marrow can contain up to 95% of haemoglobin-synthesizing cells at various stages of differentiation (Denton & Arnstein, 1973). These stages are designated pronormoblast, basophilic normoblast, polychromatic normoblast, orthochromatic normoblast and reticulocyte in order of

increasing maturity (Wintrobe, 1967). All these cells are found in the bone marrow. Although the reticulocyte occurs at 0.1–1.0% in normal individuals, during phenylhydrazine-induced reticulocytosis the blood can contain up to 90% reticulocytes, with a correspondingly low erythrocyte count.

In the present study we have characterized the membrane components of rabbit erythroid cells and show that circulating erythroid cells and marrow-bound nucleated erythroid cells have unique and mutually exclusive groups of surface-membrane components.

Materials and Methods

¹²⁵I was obtained as carrier-free Na¹²⁵I from The Radiochemical Centre, Amersham, Bucks., U.K. Glucose oxidase was obtained from Sigma (London) Chemical Co., London S.W.6, U.K., and Ficoll from Pharmacia (G.B.) Ltd., London W.5, U.K. Phosphate-buffered saline was prepared as described by Denton & Arnstein (1973).

Preparation of cells

Normal healthy adult New Zealand White rabbits were bled from the lateral ear vein and the blood was collected into acid/citrate/dextrose as an anticoagulant (Dacie & Lewis, 1975). The erythrocytes were

washed four times in iso-osmotic 0.15M-NaCl before labelling with lactoperoxidase or the preparation of 'ghosts'.

Reticulocytes were prepared by subcutaneous injection of 6mg of phenylhydrazine hydrochloride in iso-osmotic NaCl/kg body wt. for 5 consecutive days. On the sixth day, blood was collected into acid/citrate/dextrose and the cells were washed four times in iso-osmotic NaCl. Reticulocytes were estimated by supravital staining (Dacie & Lewis, 1975), and as a routine were found to be 0.1% in normal blood and 75–95% in blood from phenylhydrazine-treated animals.

Preparation of rabbit marrow cells

Adult New Zealand White rabbits were made anaemic with phenylhydrazine as described above. The animals were left for 2 days to produce an optimal reticulocytosis, and then killed by exsanguination. The marrow was removed from the long leg bones with a spatula and was suspended by forcing through butter-muslin into phosphate-buffered saline solution. The fat and most of the non-erythroid cells were removed by filtration through packed fibrous cottonwool in phosphate-buffered saline (Diepenhorst *et al.*, 1972). The erythroid-cell suspension was then washed twice in phosphate-buffered saline by centrifugation at the bench. Cells were resuspended in phosphate-buffered saline by means of a silicone-coated Pasteur pipette.

Lactoperoxidase labelling of whole cells

Erythrocytes and reticulocytes were labelled with lactoperoxidase as previously described by Boxer *et al.* (1974); 2×10^8 bone-marrow erythroid cells were suspended in 50 μ l of 310 mosm-sodium phosphate buffer, pH 7.4, 50 μ l of lactoperoxidase (10 mg/ml), 20 μ l of 60 mM-glucose, 5 μ l of butylated hydroxytoluene (0.005%, w/v), 1 μ l of 0.3 mM-KI and 0.5 mCi of Na¹²⁵I. The reaction was started by the addition of 0.005 unit of glucose oxidase and left to incubate for 30 min at room temperature (20°C). The reaction was stopped by the addition of 10 ml of cold phosphate-buffered saline and the cells were washed once in this solution and then twice more in phosphate-buffered saline containing 3% (v/v) rabbit serum. Some 90% or more of the cells excluded Trypan Blue before and after labelling.

Velocity sedimentation

Cells were separated on a size (i.e. age) basis by a modification of the method of Miller & Phillips (1969). The separation vessel had an internal diameter of 12.5 cm, and the gradient was 700 ml of 15–30% rabbit serum in phosphate-buffered saline with an initial step of 60 ml of phosphate-buffered saline containing 5–15% rabbit serum. For some experiments the concentrated side of the gradient

was supplemented with 0.1% Ficoll and the rabbit serum content was lowered to 15%. This did not affect the quality of the separation.

Cells (2×10^8 , in 20 ml of 3% rabbit serum in phosphate-buffered saline) were separated by velocity sedimentation at 1g for 3.5 h at room temperature. After the separation, the first 150 ml of gradient was discarded and then 10 ml fractions were collected on an LKB fraction collector in plastic 10 ml tubes. Cell counts were done manually on the fractions in a haemocytometer: 75–85% of the cells in the final pooled fractions excluded Trypan Blue. The pooled cells were washed twice with phosphate-buffered saline, by centrifugation at 900g for 2 min. The packed cells were lysed by suspension in 1 ml of cold water and homogenized by five passes at 1200 rev./min in a loose-fitting Potter–Elvehjem homogenizer. After centrifugation at 15000g for 2 min in an Eppendorf micro-centrifuge, the pellet was suspended in a little water and dissolved in SDS* as described below for plasma membranes.

Identification of cells

After the fractions separated by velocity sedimentation were pooled, a known number of bone-marrow cells from each fraction were smeared on a slide, fixed with methanol, stained with May–Grunwald's stain and counter-stained with Giemsa's stain (Dacie & Lewis, 1975). Cells were identified by the size and density of the staining and counts were made of the relative number of each type of bone-marrow erythroid cell. Whole bone marrow generally contained approx. 5–10% eosinophils, whereas only the fastest-moving fraction separated by velocity sedimentation contained significant numbers of these cells (10–20%).

Preparation of plasma membranes

Erythrocyte 'ghosts' were prepared by the method of Dodge *et al.* (1963). Reticulocyte 'ghosts' were prepared as partially purified 'ghosts' by the method of Lodish & Small (1975). Washed bone-marrow cells were resuspended in 0.25 M-sucrose containing 10 mM-Tris/HCl and 1 mM-EDTA, pH 7.5. The cells were broken by five passes at 1200 rev./min in a Potter–Elvehjem homogenizer with a close-fitting Teflon pestle. The homogenate was floated on an equal volume of 0.45 M-sucrose and then centrifuged at 15000g for 24 s in an Eppendorf micro-centrifuge to remove any unbroken cells and nuclei. The clear red supernatant was discarded and the plasma membranes were collected from the interface and resuspended in 0.25 M-sucrose containing 10 mM-Tris/HCl and 1 mM-EDTA, pH 7.5. This suspension was recentrifuged at 15000g for 2 min in the Eppendorf microcentrifuge.

* Abbreviation: SDS, sodium dodecyl sulphate.

Fig. 1. SDS/polyacrylamide-gel electrophoresis of rabbit erythrocyte, reticulocyte and bone-marrow erythroid-cell plasma membranes

Scans at 550nm of plasma membranes prepared as described in the text. Electrophoresis was done on SDS/polyacrylamide gels containing 8% (w/v) acrylamide and stained for protein with Coomassie Blue. (a) Erythrocyte 'ghosts'; (b) reticulocyte 'ghosts'; (c) partially purified plasma membranes from unfractionated bone-marrow erythroid cells. Abbreviation: Hb, haemoglobin.

The creamy fluffy plasma-membrane pellet was carefully removed with a silicone-coated Pasteur pipette, but the hard 'button' was discarded. This centrifugation step was repeated twice, any hard 'button' obtained being discarded, but the fluffy superficial plasma-membrane layer being retained.

The partially purified plasma membranes were then solubilized in 2vol. of 5% (w/v) SDS/5mM-EDTA/10% (v/v) glycerol/10% (v/v) mercaptoethanol/2mM-phenylmethanesulphonyl fluoride/20mM-Tris/acetate, pH8.0, and heated in a boiling-water bath for 2min.

SDS/polyacrylamide-gel electrophoresis

Gel electrophoresis was done as described by Fairbanks *et al.* (1971) in the presence of 0.1% SDS. For comparison of membrane proteins and glycoproteins, small (0.6 cm diam.) 8% polyacrylamide gels were used. The gels were stained for protein either with Coomassie Brilliant Blue, or by the periodic acid/Schiff method (Zacharias *et al.*, 1969) for carbohydrate- and particularly sialic acid-bearing proteins. To identify lactoperoxidase-labelled proteins and for lectin-binding studies, large (1.1 cm diam.) 8% polyacrylamide gels were used. After electrophoresis, gels of lactoperoxidase-labelled membranes were sliced longitudinally, stained, dried and radioautographed (Boxer *et al.*, 1974). For lectin-binding studies on membrane glycoproteins, longitudinal gel slices were used. Lectins were prepared, radio-labelled and incubated with gel slices as described by Tanner & Anstee (1976). After incubation the gel slices were stained for protein, dried and radioautographed to detect the lectin-binding bands.

Results

Proteins and glycoproteins of the membranes of circulating rabbit reticulocytes and erythrocytes

The mature rabbit erythrocyte membrane contained seven major proteins which could be detected by Coomassie Blue staining after SDS/polyacrylamide-gel electrophoresis (Fig. 1*a*). These had molecular weights of 250 000, 90 000, 75 000, 39 000, 33 000, 28 000 and 23 000, and were comparable with the proteins of the human erythrocyte membrane (Steck, 1974). The similarities between the two membranes were considerable, and we adopted the numbering system of Steck (1974) to describe the proteins of the rabbit erythrocyte membrane.

The rabbit reticulocyte membrane contained the same proteins as that of the erythrocyte, but in addition there were two reticulocyte-specific proteins, with molecular weights of 54 000 (R1) and 44 000 (R2) (Fig. 1*b*). Gels of membrane proteins from both reticulocytes and erythrocytes showed that neither cell type contained any strongly periodic acid/Schiff-staining proteins, but very faint bands with molecular weights of 90 000, 75 000 and 18 000 were observed on grossly overloaded gels when this stain was used. Although band 4 of the erythrocyte nearly always migrated as a single band, two bands of closely similar mobility were found in the region of band 4 of the reticulocyte (4 and 4A), the slower-moving of which co-migrated with erythrocyte band 4. Some preparations of erythrocyte membranes contained band 4A.

Intact rabbit reticulocytes and erythrocytes were radioiodinated by the lactoperoxidase method to compare the proteins available at the extracellular surface. In both cell types only one major protein,

Fig. 2. Polyacrylamide-gel electrophoresis of membranes from lactoperoxidase-radioiodinated rabbit erythrocytes and reticulocytes

Scans at 550 nm of radioautographs of 'ghosts' obtained from lactoperoxidase-labelled normal and enzyme-treated rabbit erythrocytes and reticulocytes. 'Ghost' proteins were separated by electrophoresis on SDS/polyacrylamide gels containing 8% (w/v) acrylamide. (a) 'Ghosts' from lactoperoxidase-labelled untreated cells. (b) 'Ghosts' from lactoperoxidase-labelled trypsin-treated cells. (c) 'Ghosts' from trypsin-treated cells which were labelled with lactoperoxidase and treated with thermolysin before haemolysis. E = erythrocyte, R = reticulocyte.

polypeptide 3, was labelled, but small amounts of radioactivity were also found in other membrane components (bands G1, G2 and G3; Fig. 2*a*). Polypeptide 3 was isolated from lactoperoxidase-labelled rabbit erythrocytes and reticulocytes, and peptide 'maps' were prepared from thermolysin digests of the

Fig. 3. Binding of radioiodinated lectins to membrane components of rabbit erythrocytes, reticulocytes and bone-marrow erythroid cells

The components of plasma membranes of rabbit erythrocytes, reticulocytes and bone-marrow erythroid cells, prepared as described in the text, were separated by SDS/polyacrylamide-gel electrophoresis on gels containing 8% (w/v) acrylamide. Centre slices from the gels were incubated with radioiodinated lectins as described by Tanner & Anstee (1976). Scans at 550nm are presented of radioautographs of dried gel slices after incubation with: (a) 0.16mg of concanavalin A (containing 7×10^6 c.p.m. of ^{125}I); (b) 0.25mg of the lectin from *Phaseolus vulgaris* (containing 2.5×10^6 c.p.m. of ^{125}I); (c) 0.5 ml of the labelled lectin from *Maclura aurantiaca* (containing 7×10^4 c.p.m. of ^{125}I /ml), which had an agglutinin titre of 1:8; (d) 0.5 ml of wheat-germ agglutinin (containing 5×10^6 c.p.m. of ^{125}I /ml), which had a haemagglutination titre of 1:256. In each case (i) represents erythrocyte 'ghosts', (ii) represents reticulocyte 'ghosts' and (iii) represents partially purified plasma membranes from unfractionated bone-marrow erythroid cells. In control experiments with concanavalin A and the lectin from *M. aurantiaca*, gel slices were incubated with the radioiodinated lectin and the appropriate monosaccharide inhibitor (Tanner & Anstee, 1976). In these cases no binding of radioiodinated lectin was observed. The erythrocyte and reticulocyte samples were run in parallel on a separate occasion to the erythroid-cell plasma membranes, and thus the former gels are not directly comparable with the latter.

Table 1. *Partial purification of rabbit bone-marrow erythroid-cell plasma membranes*

Fraction	Plasma membrane				Endoplasmic reticulum				Lysosomes		Mitochondria	
	5'-Nucleotidase				Glucose 6-phosphatase				Acid phosphatase		Glutamate dehydrogenase	
	Sp. activity	Recovery (%)	Rel. sp. radioactivity	¹²⁵ I Recovery (%)	Sp. activity	Recovery (%)	Sp. activity	Recovery (%)	Sp. activity	Recovery (%)	Sp. activity	Recovery (%)
Homogenate	0.6 ± 0.02	100	1.0	100	3.05 ± 0.1	100	0.7 ± 0.1	100	1.45 ± 0.1	100	nt	100
1st supernatant	0.2 ± 0.06	40 ± 7	0.6 ± 0.1	39 ± 5	6.8 ± 0.8	63 ± 3	1.2 ± 0.1	83 ± 13	nt	nt	nt	nt
Interface	3.1 ± 0.4	33 ± 9	2.3	48	1.65 ± 0.2	13 ± 3	0.51 ± 0.14	14 ± 6	1.0 ± 0.1	7 ± 1.5	1.0 ± 0.1	7 ± 1.5
*Plasma-membrane layer of 1st pellet	5.5 ± 0.6	31 ± 6	3.0 ± 0.5	23 ± 8	nt	nt	0.30 ± 0.04	7	0.42 ± 0.2	2 ± 1.5	0.3 ± 0.1	1.5 ± 1.5
Crude plasma membranes	6.2 ± 0.2	22 ± 5	3.2 ± 0.6	18 ± 11	0.9 ± 0.1	4 ± 1	0.18 ± 0.05	4 ± 1	0.3 ± 0.1	1.5 ± 1.5	0.3 ± 0.1	1.5 ± 1.5

* This refers to the superficial plasma-membrane-containing layer (excluding the hard pellet) obtained on recentrifugation (30000 g-min) of the plasma-membrane layer (excluding the hard pellet) from the material sedimented on centrifugation of the interface obtained from the initial sucrose-cushion centrifugation.

Enzyme activities were assayed in duplicate as described by Belfield & Goldberg (1968) for 5'-nucleotidase (data from three preparations); Swanson (1955) for glucose 6-phosphatase by using the P_i assay of Baginski *et al.* (1967) (data from three preparations); Delbrück *et al.* (1959), as modified by Frieden (1959) for glutamate dehydrogenase (data from two preparations). Acid phosphatase was assayed in a reaction mixture containing 10 mM-sodium acetate, adjusted to pH 5.0 with acetic acid, and 1 mM-nitrophenyl phosphate. The enzyme-containing fractions were diluted tenfold with water and 0.2 ml of this sample was added to 1 ml of the reaction mixture. After incubation at 37°C for 10 min, 0.1 ml of 1 M-NaOH was added, and after removal of the membranous material by centrifugation (30000 g-min) in the Eppendorf micro-centrifuge the A_{400} of the supernatant was determined. Activities were calculated by means of an extinction coefficient of $1.83 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Trentham & Gutfreund, 1968). The data from three preparations are given. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. ^{125}I was used as a plasma-membrane marker and was covalently bound to the surface of intact bone-marrow erythroid cells by the lactoperoxidase method described in the text. ^{125}I radioactivity was determined by scintillation counting. Data from three preparations are given and the results were compared by setting the specific radioactivity in the homogenate from each experiment to 1.0. The activity of 5'-nucleotidase is expressed in arbitrary units/min. The results are expressed as means $\pm$ S.D., except where they are based on a single preparation. nt, Not tested.

proteins as previously described (Boxer *et al.*, 1974). Both rabbit reticulocyte and rabbit erythrocyte polypeptide 3 gave the same six major and six minor labelled peptides. These results suggested that there was no difference in the accessibility of polypeptide 3 to lactoperoxidase labelling at the outer surface of the rabbit reticulocyte and erythrocyte. It is noteworthy that four of the labelled peptides in rabbit polypeptide 3 had a similar mobility on peptide 'maps' to the major labelled peptides found in the human protein by Boxer *et al.* (1974).

When intact rabbit reticulocytes and erythrocytes were treated with thermolysin, polypeptide 3 was cleaved in both cases to yield the same membrane-bound fragment of mol.wt. 60000 (3f; Fig. 2c). However, polypeptide 3 was relatively resistant to trypsin cleavage under similar conditions, although a very small amount of membrane-bound peptide of the same molecular weight as that obtained from thermolysin treatment was found in this case (Fig. 2b).

The binding of lectins to the membrane glycoproteins of rabbit reticulocytes and erythrocytes was also studied. Radiolabelled concanavalin A and the lectin from *Phaseolus vulgaris* bind to the same glycoproteins in both rabbit reticulocyte and erythrocyte membranes, as shown by separation by SDS/polyacrylamide-gel electrophoresis (Figs. 3a and 3b). These proteins had molecular weights of 90000, 43000, 34000 and 24000 (co-migrating with bands 3, G1, G2 and G3). No significant binding of the lectins from *Maclura aurantiaca* and wheat germ was observed.

Proteins and glycoproteins of plasma membranes prepared from unfractionated rabbit bone-marrow erythroid cells

Crude plasma-membrane fractions were prepared

from the bone marrow of anaemic rabbits which contained 75–95% nucleated erythroid cells. Five generally accepted markers were used to monitor the plasma-membrane fractionation procedure. 5'-Nucleotidase and the radioactivity of samples after radioiodination by lactoperoxidase of intact bone-marrow cells were both used as plasma-membrane markers. Glucose 6-phosphatase, acid phosphatase and glutamate dehydrogenase were used as markers for the microsomal fraction, lysosomal contamination and mitochondrial contamination respectively. Table 1 shows the activity and recovery of these markers at various stages in the fractionation procedure for several preparations. These data indicate that, although the final preparations were not entirely pure, they predominantly consisted of plasma membranes and were not substantially contaminated by other organelle membranes. The proteins of these plasma membranes were separated by SDS/polyacrylamide-gel electrophoresis (Fig. 1c). We have designated these proteins A–I in order of increasing electrophoretic mobility, and their apparent molecular weights and relative abundance are shown in Table 2.

The surface proteins of intact unfractionated erythroid cells from bone marrow containing 15% non-nucleated cells (from anaemic rabbits) were radioiodinated by the lactoperoxidase method. The pattern of labelling of proteins in the plasma membranes prepared from these cells is shown in Fig. 4(b). Proteins A, G and H were strongly labelled, and less label was found in proteins B, C, D and E. A small amount of labelling was observed in the position expected for polypeptide 3 (the major component of the membrane of non-nucleated erythroid cells). When intact erythroid cells from normal rabbit bone marrow (containing 30% non-nucleated cells) were similarly radioiodinated, a higher proportion of

Table 2. *Molecular weights of the proteins of rabbit erythroid-cell plasma membranes*

Subunit molecular weights were determined by SDS/polyacrylamide-gel electrophoresis on gels containing 8% acrylamide. Molecular-weight standard proteins were ovotransferrin (77000), bovine serum albumin (67000), glutamate dehydrogenase (56000), lactate dehydrogenase (36000), penicillinase (28500), haemoglobin (16000) and cytochrome *c* (13000). The percentage of total stain taken up by each protein was estimated from the areas under each peak of scans at 550nm of the polyacrylamide gels.

Erythrocytes		Reticulocytes		Bone-marrow erythroid cells		
Protein	Mol.wt.	Protein	Mol.wt.	Protein	Mol.wt.	Percentage of total stain
Spectrin	250000	Spectrin	250000	A	80000	1.1
3	90000	3	90000	B	54000	0.6
4	65000	4	75000	C	49000	1.2
—	—	R1	54000	C'	44000	0.8
—	—	R2	44000	D	32000	13.2
5	39000	5	39000	E	29000	5.6
6	36000	6	36000	F	24000	2.1
7	28000	7	28000	G	18000	42.4
8	23000	8	23000	H	16000	19.1
—	—	—	—	I	14000	13.9

Fig. 4. Polyacrylamide-gel electrophoresis of partially purified plasma membranes from lactoperoxidase-labelled mixed bone-marrow erythroid cells

Scans at 550nm of radioautographs obtained from the plasma membranes of labelled mixed bone-marrow erythroid cells. Plasma-membrane proteins were separated by electrophoresis on SDS/polyacrylamide gels containing 8% (w/v) acrylamide. (a) Mixed bone-marrow erythroid cells containing 30% non-nucleated cells from a normal rabbit; (b) mixed bone-marrow erythroid cells containing 15% non-nucleated cells from an anaemic rabbit.

labelled polypeptide 3 was observed in the plasma membranes (Fig. 4a). Thus the small amount of polypeptide 3 observed in the bone-marrow erythroid-cell plasma-membrane preparations was probably derived from non-nucleated cells in the bone marrow. It is not clear whether these were bone-marrow-bound reticulocytes or circulating cells, which always unavoidably contaminated bone-marrow samples. Interestingly, none of the other labelled bands in these plasma-membrane preparations corresponded to the membrane components of reticulocytes or erythrocytes which could be labelled with lactoperoxidase (Fig. 2a).

The binding of concanavalin A, wheat-germ agglutinin and the lectins from *P. vulgaris* and *M. aurantiaca* to bone-marrow erythroid-cell plasma-membrane components is shown in Fig. 3. Each lectin gave a distinct spectrum of binding, and Table 3 summarizes these results. Bands A, G and H bound all these lectins. All of the protein components of bone-marrow erythroid-cell plasma membranes, except bands F and I, bound lectins and could be labelled with lactoperoxidase in the intact cell.

Table 3. Summary of lectin binding by glycoproteins of the rabbit bone-marrow cell plasma membranes

This Table summarizes the data in Fig. 3. + indicates binding of radioiodinated lectin and - indicates absence of binding.

Glyco-protein	Concana-valin A	<i>P. vulgaris</i>	<i>M. aurantiaca</i>	Wheat-germ agglutinin
A	+	+	+	+
B	+	+	-	+
C	+	-	-	-
C'	+	-	-	-
D	-	+	+	+
E	+	-	-	-
G	+	+	+	+
H	+	+	+	+

Surface proteins and glycoproteins of separated bone-marrow cells

Since bone-marrow-bound and circulating erythroid cells appeared to have distinct plasma-membrane components, we studied the membranes of the different developmental stages of erythroid

Fig. 5. Separation into age groups of lactoperoxidase-labelled bone-marrow erythroid cells by velocity sedimentation at 1g

(a) Approx. 2×10^8 bone-marrow erythroid cells which had been previously labelled with lactoperoxidase were separated into age groups by velocity sedimentation at 1g in a rabbit serum gradient at 25°C by the method of Miller & Phillips (1969) (see the Materials and Methods section). The cells were pooled into four fractions as indicated in the Figure. (b) Distribution of cell types in fractions collected after velocity-sedimentation separation of rabbit bone-marrow erythrocyte cells. Lactoperoxidase-labelled rabbit bone-marrow erythrocyte cells were separated into age groups by velocity sedimentation as described in the text. Fractions were pooled (a) and examined histologically by using the May-Grunwald-Giemsa stain. Cell counts were made of the relative numbers in each fraction of non-nucleated cells ($\square$), orthochromatic normoblasts ($\circ$), and all other erythroid cells more immature than the orthochromatic normoblast (Δ). The contamination by eosinophils did not exceed 10% in any fraction.

Table 4. Velocity sedimentation of rabbit bone-marrow erythroid cells

The Table summarizes data from four experiments. An index of the average age of the cells in each fraction collected after velocity sedimentation was calculated by allotting 'scores' to each cell type. Thus the 'average age index' of the cells in any one fraction is equal to $[(\% \text{ pronormoblasts} \times 1) + (\% \text{ intermediate normoblasts} \times 2) + (\% \text{ orthochromatic normoblasts} \times 3) + (\% \text{ non-nucleated cells} \times 4)]/100$. Intermediate normoblasts are taken to be basophilic and polychromatic normoblasts.

Fraction no.	Expt. no.	Average age index			
		1	2	3	4
1		1.3	1.8	1.4	1.1
2		1.8	1.9	1.7	1.9
3		2.5	2.1	1.9	2.0
4		3.1	2.4	2.6	2.8
5		3.8	2.9	—	—

cells which were present in the bone marrow to establish the point at which this changeover of surface components occurs.

Denton & Arnstein (1973) have shown that rabbit bone-marrow erythroid cells may be separated according to their developmental age by velocity sedimentation at 1g. We have used this method with the apparatus described by Miller & Phillips (1969). Typical results obtained by this separation method are shown in Figs. 5 and 7. Because of differences in the initial distribution of erythroid cell types in individual bone-marrow samples, the sedimentation profile showed some variation in each case; however, the results of four separations, given in Table 4, showed that the cells were reproducibly separated according to their age.

Since only very small amounts of cells could be dealt with by this method, two approaches were used to investigate the surface-membrane proteins of these cells. In the first approach the surface components of marrow erythroid cells from an anaemic rabbit were radioiodinated by the lactoperoxidase method and then separated into age groups (Fig. 5). Fractions were pooled, and, after a lysis step, the membrane proteins were separated by SDS/polyacrylamide-gel electrophoresis. Fig. 5 also shows the distribution of cell types in the fractions. Densitometric scans of the radioautographs of the radioiodinated components present in each cell fraction are shown in Fig. 6. Traces of labelled polypeptide 3 were found in all fractions, in amounts which seemed to correlate with the number of non-nucleated cells in each fraction. A second similar experiment (results not shown) gave results which supported this conclusion.

In the second approach, bone-marrow erythroid cells from an anaemic rabbit were separated by

Fig. 6. Polyacrylamide-gel electrophoresis of plasma membranes derived from the pooled fractions obtained after velocity sedimentation of lactoperoxidase-labelled bone-marrow erythroid cells

Scans at 550nm of radioautographs obtained from membrane fractions of the cells in the pooled fractions collected after velocity-sedimentation separation of lactoperoxidase-labelled bone-marrow erythroid cells (Fig. 5). The plasma-membrane components were separated by SDS/polyacrylamide-gel electrophoresis on gels containing 8% (w/v) acrylamide. Lactoperoxidase-labelled plasma-membrane components from cells in (i) fraction 1, (ii) fraction 2, (iii) fraction 3 and (iv) fraction 4.

Fig. 7. Separation into age groups of bone-marrow erythroid cells by velocity sedimentation

(a) Approx. 2×10^8 bone-marrow erythroid cells were separated into age groups by velocity sedimentation at 1 g in a rabbit serum gradient at 25°C (see the text). The cells were pooled into five fractions as indicated. (b) Distribution of cell types in fractions collected after velocity-sedimentation separation of rabbit bone-marrow erythroid cells. Untreated rabbit bone-marrow erythroid cells were separated into age groups by velocity sedimentation as described in the text and then pooled into five fractions. A sample from each fraction was smeared, stained with May-Grunwald-Giemsa stain, and examined microscopically. Cell counts were made of the relative numbers in each fraction of non-nucleated cells (□), orthochromatic normoblasts (○) and all other erythroid cells present before the orthochromatic normoblast stage (△). Each fraction contained less than 7% eosinophils.

velocity sedimentation. The cell sedimentation profile shown in Fig. 7 was obtained, fractions were pooled in the manner indicated and the slowest-moving debris-rich fraction was discarded. Fig. 7 also

shows the distribution of cell types in each fraction. The binding of concanavalin A, the lectin from *P. vulgaris*, wheat-germ agglutinin and the lectin from *M. aurantiaca* to the components of the membrane-rich pellet obtained after lysis of each fraction was studied. Each cell fraction gave the same pattern of lectin-binding components, and these were similar to those obtained from plasma membranes of unfractionated bone-marrow erythroid cells (Fig. 3). No binding of concanavalin A or the lectin from *P. vulgaris* was observed in a position equivalent to band 3 in any of the fractions.

Discussion

Our results obtained by using SDS/polyacrylamide-gel electrophoresis and lactoperoxidase labelling show that both rabbit reticulocytes and erythrocytes contain one major component accessible at the external surface, polypeptide 3. These results confirm those of Lodish & Small (1975). No differences between reticulocyte and erythrocyte polypeptide 3 could be detected and the rabbit protein appeared similar in many ways to the corresponding protein of the human erythrocyte membrane. The behaviour, on proteolysis, of polypeptide 3 was also shown to be strikingly similar to that of the equivalent human erythrocyte-membrane protein. However, the remaining surface components of rabbit erythrocytes differed from those of the human erythrocyte. The erythrocytes of the variety of rabbit that we studied lacked the major membrane sialoglycoprotein and the other periodic acid/Schiff-staining glycoproteins found in the human erythrocyte (Steck, 1974) and erythrocytes of many other species (Carraway, 1975). This conclusion was confirmed by our studies of the lectin receptors and the lactoperoxidase-labelling characteristics of rabbit erythrocyte-membrane proteins. These experiments did show, however, that a number of other minor glycoproteins exist in the rabbit erythrocyte cell membrane, and these may replace the periodate/Schiff-staining components found in the erythrocytes of other species.

We have used a crude plasma-membrane preparation to study the surface components of unfractionated rabbit bone-marrow erythroid cells. The proteins in this preparation (except bands F and I) could be labelled with ¹²⁵I by the lactoperoxidase method in intact cells, and were shown to bind lectins. Both of these results, as well as those obtained by using plasma-membrane markers (Table 1), are consistent with our conclusion that the proteins in this preparation were derived from the plasma membranes of these cells. Further support comes from our experiments with cells separated by velocity sedimentation, in which the membrane-containing fraction was prepared by a simple lysis step. The electrophoretic mobility of the proteins radioiodinated by lacto-

peroxidase and their lectin-binding properties were the same as those of the more highly purified plasma-membrane preparations from unfractionated bone-marrow cells.

The set of proteins found in bone-marrow erythroid-cell plasma membranes is distinctly different from that found in the membrane of the circulating erythroid cell. Fehlmann *et al.* (1975) reported no difference in the Coomassie Blue-staining pattern of membrane proteins from rabbit erythrocytes, reticulocytes and bone-marrow erythroid cells separated by SDS/polyacrylamide-gel electrophoresis. However, the gel pattern that they obtained from the rabbit erythrocyte membrane bears little or no resemblance to the well-known characteristic separation pattern for rabbit erythrocyte membranes obtained by us and other workers (Lodish & Small, 1975; Koch *et al.*, 1975).

Although no bands were obtained from polyacrylamide gels of bone-marrow erythroid-cell plasma-membrane proteins when stained by the periodic acid/Schiff method, the results of the lectin-binding experiments clearly indicated that eight of the ten protein-staining bands in the plasma-membrane preparations are glycoproteins. Since plasma-membrane carbohydrates are generally located at the extracellular surface, these results suggested that all of these glycoproteins are exposed at the outer surface of the cells, confirming the results of the lactoperoxidase labelling of intact bone-marrow erythroid cells. It is perhaps surprising that only bands F and I are not exposed to the external milieu of the cell.

The absence of polypeptide 3, the major erythrocyte-membrane protein, from the protein-stained polyacrylamide gels of plasma membranes derived from unfractionated rabbit bone-marrow erythroid cells, and the results of examination of the surface components of the cells by using lactoperoxidase labelling, suggested that this protein may be absent from or present in only small quantities in the plasma membranes of nucleated erythroid cells. The difference in membrane components between nucleated and non-nucleated rabbit erythroid cells suggests that the maturation of the erythroid cell involves a complete change in the membrane proteins and glycoproteins by the time the circulating stage of the cell is reached. Although we have not been able to separate erythroid cells into homogeneous populations by the velocity-sedimentation method, fractions substantially enriched in the cell types of interest were always obtained, allowing us to correlate changes in membrane components with the appearance of cell types in the fractions. Polypeptide 3 is involved in the transport of anions across the erythrocyte membrane (Cabantchik & Rothstein, 1974). Since bone-marrow-bound nucleated erythroid cells are unlikely to need to equilibrate anions at a rate

as fast as erythrocytes, the absence of polypeptide 3 from bone-marrow-bound cells is not altogether surprising.

When bone-marrow erythroid cells which had been previously radioiodinated were separated into age groups, it was observed that each fraction contained an amount of labelled polypeptide 3 which seemed to correspond to the number of non-nucleated cells in that fraction. This further supports the conclusion that nucleated bone-marrow erythroid cells contain little or no plasma-membrane polypeptide 3. The pattern of lectin binding for similar fractions obtained in a second experiment confirmed this result. The data in Fig. 6 also showed that the stages of the erythroid-cell series up to and including the orthochromatic normoblast contain the same set of surface components.

All the data presented here indicate the presence of distinct sets of glycoproteins in the membranes of nucleated marrow-bound erythroid cells and circulating erythroid cells. A major functional difference between these two groups of cells is that one group is tissue-bound, whereas the other group circulates freely. At some stage of the erythroid cell's development the set of surface sites governing its ability to bind to other bone-marrow constituents must be altered in such a way as to allow the release of the cell, after the loss of its nucleus, from the sinusoids of the bone marrow into the bloodstream. The dramatic difference that we found between the surface components of the marrow-bound and circulating erythroid cells suggests that this change is accompanied by the loss of tissue-bound cell-surface components and the acquisition of new circulating cell-surface components. This alteration in surface components occurs after the orthochromatic-normoblast stage of the erythroid cell and must be very close to the point at which erythroid cells are released from the bone marrow.

The cells undergoing this change in surface components are all histochemically classified as reticulocytes. It is clear that these cells must be heterogeneous with respect to their surface components, containing both a group of cells with circulating surface components and a group of cells with predominantly tissue-bound surface components. The sequential loss of tissue-bound surface components and gain of circulating surface components seems unlikely, since this would necessitate a cell stage with no surface components. This suggests that a transitional reticulocyte stage must exist at which there is the concurrent loss of tissue-bound surface components and synthesis of circulating components. We have not been able to detect cells at this transitional stage.

In the human, four groups of reticulocytes (I-IV) have been histochemically defined on the basis of the amount of reticulum found within them (Wickramasinghe, 1975). In the normal human

Fig. 8. Schematic representation of the 'changeover' of plasma-membrane components during rabbit erythroid-cell maturation

The diagram represents the postulated disappearance of tissue-bound membrane sites and the concomitant synthesis and insertion into the membrane of circulating sites during the orthochromatic-normoblast and reticulocyte stages of the erythroid-cell line. Abbreviations: PrN, pronormoblast; BN, basophilic normoblast; PN, polychromatic normoblast; ON, orthochromatic normoblast. The vertical scale of units is arbitrary and no comparison of the relative amounts of membrane components in the marrow-bound and circulating species is intended. The hatched bar represents the region in which the transition from marrow to circulating cell occurs. The actual point of the transition appears to depend on the haematological state of the erythroid system of the individual (see the Results and Discussion sections).

adult the most immature stages (I and II) are predominantly found in the bone marrow, and the least reticulated stages (III and IV) are mainly found in the circulation. However, in anaemic individuals responding to treatment, type I and II reticulocytes are released into the circulation during the first high reticulocyte response and decrease in number in the circulation as the anaemia is checked (Wintrobe, 1967). Similarly, we have observed significant numbers of stage-I and -II reticulocytes in the blood of anaemic rabbits (typically 16% and 20% respectively of the total reticulocyte count). Correspondingly fewer reticulocytes are found in the bone marrow of anaemic rabbits than in the normal bone marrow (Denton & Arnstein, 1973). Thus the appearance of the most immature form of reticulocyte in the circulation suggests that, under these conditions, release from the bone marrow occurs almost concurrently with the loss of the nucleus from the orthochromatic normoblast. Tavassoli & Crosby (1973) have previously suggested that this may indeed be the case. It seems likely that the biochemical restraints acting on the orthochromatic normoblast

to prevent its escape from the bone marrow, i.e. the presence of tissue-bound surface components, are rapidly lost during the enucleation process. Fig. 8 summarizes the changes in the surface components of the erythroid-cell series and relates these changes to the histochemically defined stages of erythropoiesis.

The extent of the compartment of reticulocytes containing only tissue-bound surface components is not clear, as we have not been able to isolate cells at this stage. If the onset of the synthesis of circulating components is linked to the loss of the nucleus from the orthochromatic normoblast, this stage may effectively be non-existent. It is probable that in normal bone marrow the compartment of reticulocytes containing only tissue-bound membrane components is larger and may form a significant proportion of bone-marrow reticulocytes.

Polypeptide 3 is the major carrier of protein-bound carbohydrate in the rabbit erythrocyte. Since none of the surface components of the rabbit erythrocyte is found in nucleated rabbit bone-marrow cells, we can conclude that the carbohydrates bound to polypeptide 3 and the other surface glycoproteins of the mature rabbit erythrocyte play no part in the cell-differentiation processes which lead to the orthochromatic normoblast. This carbohydrate may have roles in the dissociation of the reticulocyte from the bone marrow, the circulating phase of the erythrocyte and the turnover of senescent erythrocytes. It seems reasonable to draw a similar conclusion with regard to the protein-bound carbohydrate of the human erythrocyte. Although the human erythrocyte contains three glycoproteins which are not found in the rabbit erythrocyte, individuals with erythrocytes which lack the major sialoglycoprotein have an apparently normal haematology (Tanner & Anstee, 1976; Anstee *et al.*, 1977).

After the extrusion of the nucleus from the orthochromatic normoblast outside the sinusoids of the bone marrow, the newly formed reticulocytes usually pass through gaps in the walls of the sinusoids by diapedesis (Weiss, 1965). Although the morphology of this process is well characterized, the biochemical changes involved are less well understood. Our results suggest that cell-surface components are important in the change in cellular interactions which occurs during this process.

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References

- Anstee, D. J., Barker, D. M., Judson, P. A. & Tanner, M. J. A. (1977) *Br. J. Haematol.* **35**, 309-320
- Baginski, E. S., Zak, B. & Foa, P. P. (1967) *Clin. Chim. Acta* **15**, 155-158

- Belfield, A. & Goldberg, D. M. (1968) *Nature (London)* **219**, 73–74
- Boxer, D., Jenkins, R. & Tanner, M. J. A. (1974) *Biochem. J.* **137**, 531–534
- Cabantchik, Z. I. & Rothstein, A. (1974) *J. Membr. Biol.* **15**, 207–226
- Carraway, K. L. (1975) *Biochim. Biophys. Acta* **415**, 379–410
- Dacie, J. V. & Lewis, S. M. (1975) *Practical Haematology*, Churchill-Livingstone, London
- Delbrück, A., Zebe, E. & Bücher, Th. (1959) *Biochem. Z.* **331**, 273–280
- Denton, M. J. & Arnstein, H. R. V. (1973) *Br. J. Haematol.* **24**, 7–17
- Diepenhorst, P., Sprokholt, R. & Prins, H. K. (1972) *Vox Sang.* **23**, 308–320
- Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100**, 119–130
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
- Fehlmann, M., Lafleur, L. & Marceau, N. (1975) *Biochem. Biophys. Res. Commun.* **66**, 322–327
- Frieden, C. (1959) *J. Biol. Chem.* **234**, 809–820
- Koch, P. A., Gardner, F., Gartrell, J. & Carter, J. (1975) *Biochim. Biophys. Acta* **389**, 177–187
- Lodish, H. F. & Small, B. (1975) *J. Cell Biol.* **65**, 51–64
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Miller, R. G. & Phillips, R. A. (1969) *J. Cell. Physiol.* **73**, 191–202
- Oseroff, A. R., Robbins, P. W. & Burger, M. M. (1973) *Annu. Rev. Biochem.* **42**, 647–682
- Singer, S. J. (1974) *Adv. Immunol.* **19**, 1–66
- Steck, T. L. (1974) *J. Cell Biol.* **62**, 1–19
- Swanson, M. A. (1955) *Methods Enzymol.* **2**, 241–543
- Tanner, M. J. A. & Anstee, D. J. (1976) *Biochem. J.* **153**, 265–270
- Tavassoli, M. & Crosby, W. H. (1973) *Science* **179**, 912–913
- Trentham, D. R. & Gutfreund, H. (1968) *Biochem. J.* **106**, 455–460
- Weiss, L. (1965) *J. Morphol.* **117**, 467–537
- Wickramasinghe, S. (1975) *Human Bone Marrow*, pp. 162–232, Blackwell, London
- Wintrobe, M. M. (1967) *Clinical Hematology*, pp. 63–223, Henry Kimpton, London
- Zacharias, R. J., Zell, T. E., Morrison, J. H. & Woodcock, J. J. (1969) *Anal. Biochem.* **30**, 148–152