Biochem. J. (1981) **195**, 129–137
Printed in Great Britain

Synthesis of the erythrocyte anion-transport protein

Immunochemical study of its incorporation into the plasma membrane of erythroid cells

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(*Received 6 October 1980/Accepted 10 December 1980*)

We studied the surfaces of maturing rabbit bone-marrow erythroid cells for the presence of the erythrocyte anion-transport protein by using an immunochemical method. An antibody was raised against the purified anion-transport protein. The antibody was shown to react specifically with the anion-transport protein and it recognized determinants in the extracellular as well as the cytoplasmic or intramembranous domains of the protein. The binding of the antibody to the surface of intact rabbit bone-marrow erythroid cells was studied by using the *Staphylococcus aureus* 'rosette' technique described by Gahmberg, Jokinen & Andersson [(1978) *Blood* **52**, 379–386]. Although pronormoblasts had little of the protein, there was a progressive increase in the amount of the protein at the surface of cells of increasing maturity up to the reticulocyte stage. Most of the protein is inserted into the plasma membrane between the polychromatic-normoblast and reticulocyte stage of the cell.

The major integral membrane protein of the erythrocyte (Band 3; Steck, 1974) is involved in anion transport (for a review, see Cabantchik *et al.*, 1978). Previous work has suggested that the anion-transport protein is absent from nucleated bone-marrow erythroid cells, but the reticulocyte membrane contains amounts of the protein similar to that present in erythrocytes (Light & Tanner, 1977). Only the earliest reticulocyte stages have been shown to incorporate the protein into the plasma membrane (Light & Tanner, 1978).

We report here the results of a further study of the erythroid-cell stages at which the anion-transport protein becomes present at the cell surface. We have used an antibody to detect the presence of the anion-transport protein, and our results suggest that although increasing amounts of the protein are detectable in maturing nucleated stages of erythroid cells, most of the anion-transport protein is incorporated between the penultimate nucleated stage (polychromatic normoblast) and the reticulocyte stage of the cell.

Materials and methods

Blood was drawn from the lateral ear vein of New Zealand White rabbits (male), with acid/citrate/dextrose (3 vol./17 vol. of blood) or heparin (15 units/ml) as an anticoagulant.

Rabbits were made anaemic either by subcutaneous injection of phenylhydrazine hydrochloride (6 mg/kg body wt.) on 5 successive days or by five bleedings each of 30 ml over a period of 7 days. Bone-marrow erythroid cells were prepared as described previously (Light & Tanner, 1977), from rabbits (made anaemic by phenylhydrazine treatment) on day 10 after the first injection. Cells from rabbits made anaemic by bleeding were taken on day 8 after the first bleeding. In both cases the animals were injected intravenously with 1 ml of typhoid–paratyphoid A and B vaccine (Wellcome) 3 h before death (Denton & Arnstein, 1973). Cells were identified after smearing on slides by staining with May–Grunwald's stain and counter-staining with Giemsa's stain (Dacie & Lewis, 1975). Reticulocytes in the circulation were stained with New Methylene Blue (Dacie & Lewis, 1975).

Preparation of rabbit erythrocyte anion-transport protein

Erythrocyte 'ghosts' were prepared from fresh blood from normal New Zealand White rabbits as described by Dodge *et al.* (1963). The anion-transport protein was isolated by a procedure adapted from that of Steck & Yu (1973) and Yu *et al.* (1973). All operations were performed at 4°C. First 7 vol. of cold 45.6 mM-lithium 3',5'-di-iodosalicylate (BDH) in 5 mM-sodium phosphate, pH 8.0, was mixed with 1 vol. of erythrocyte 'ghosts' suspended in 5 mM-sodium phosphate, pH 8.0. The

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mixture was stirred on ice for 20min and centrifuged at 40000*g* for 45min. The pellet was suspended in 56mM-sodium borate buffer, pH8.0, and re-centrifuged. The pellet was resuspended with a Potter homogenizer, to a volume equivalent to that of the original erythrocyte 'ghosts', in 56mM-sodium borate, pH8.0, containing 0.5% Nonidet P40 (BDH) (or Triton X-100), 2% (v/v) propan-2-ol and 2mM-phenylmethanesulphonyl fluoride (Sigma) and stirred on ice for 20min. After centrifugation for 90min at 40000*g*, the supernatant was frozen in small batches in liquid N_2 and stored at −70°C.

Preparation of erythrocyte lipids

Crude rabbit and human erythrocyte lipids were prepared by a method similar to that described by Veerkamp & Broeckhuyse (1976). Erythrocyte membranes were mixed with 10vol. of methanol/chloroform (1:1, v/v), and homogenized in a Potter homogenizer. A further 8.3vol. of chloroform and 1.7vol. of methanol were added and the mixture was again homogenized. After standing for 10min, the mixture was centrifuged at 30000*g* for 20min and the supernatant was collected. The protein pellet was then re-extracted as described above and the two supernatants were combined. The supernatant was evaporated in a rotary evaporator until an aqeuous residue remained. Then 5vol. of benzene/methanol (4:1, v/v) was added to the aqueous residue and the mixture was evaporated until nearly dry. This residue was dissolved in a small volume of chloroform/methanol (2:1, v/v), dried under a stream of N_2 at room temperature, redissolved in toluene/methanol (4:1, v/v) and stored at −20°C.

Incorporation of anion-transport protein into phospholipid vesicles

A solution of anion-transport protein, prepared by extraction with Triton X-100 as described above, was made 250mM in NaCl and 0.1% in sodium deoxycholate (Maybridge Chemicals, Tintagel, Cornwall, U.K.). A mixture of phosphatidylcholine/dicetyl phosphate (9:1, w/w) was sonicated on ice by using two 15s bursts at 6A (with a Dawes Soniprobe, type 1130) and added to give a final concentration of 3mg/ml. The pH was adjusted to 7.5, and 0.3g of Bio-Beads SM-2 (Bio-Rad) was added per ml of original Triton X-100 extract to remove the detergent (Gerritsen *et al.*, 1978), and the mixture was stirred on ice for 2h. The Bio-Beads (SM-2) were removed by passing the suspension down a small glass-wool column. The vesicles were then centrifuged for 1½h at 190000*g*, and the pellet was resuspended in phosphate-buffered saline (0.135M-NaCl, containing 12mM-sodium phosphate, pH7.4), frozen in liquid N_2 and stored at −70°C.

Preparation of antiserum

Antiserum was prepared by subcutaneous injection of rabbit erythrocyte anion-transport protein preparation into a sheep. The first three injections were with 500μg of protein incorporated into sonicated phospholipid vesicles. These failed to elicit an antibody response when tested by one-dimensional 'rocket' immunoelectrophoresis and there was no increase in the agglutination titre against rabbit erythrocytes over that of the pre-immunization serum.

Subsequent immunizations (at 14-day intervals) used 500μg of anion-transport protein preparation (extracted with Nonidet P40) without prior incorporation into phospholipid vesicles, mixed with 1vol. of Freund's complete adjuvant. This resulted in an antiserum that gave a precipitin line on 'rocket' immunoelectrophoresis. The sheep was bled 8 days after the third of these injections (serum A). The same sheep was subsequently given three further immunizations of the same antigen preparation and the serum collected 14 days after the last injection (serum B).

Preparation of sheep immunoglobulin G fractions and immunoelectrophoresis

The method was modified from that of Harboe & Ingild (1976). Sheep serum (100ml) was treated with 25g of $(NH_4)_2SO_4$ and left for 20min at room temperature, before being placed in a refrigerator overnight. The creamy-white precipitate was collected by centrifugation at 4000*g* for 20min and washed twice with 1.75M-$(NH_4)_2SO_4$. The pellet was then redissolved in 30ml of distilled water, and dialysed against 5 litres of phosphate-buffered saline, pH7.4, overnight and then dialysed against 5 litres of 0.1M-NaCl containing 10mM-sodium phosphate buffer (pH7.5) for a further 3 days at 4°C. The sample was added to 25ml of Whatman DEAE-cellulose, DE-52 which had been equilibrated with 0.1M-NaCl containing 10mM-sodium phosphate, pH7.5, and stirred slowly on ice for 30min. The mixture was centrifuged and the supernatant collected. The DE-52 resin was washed with a further 25ml of the same buffer and the supernatant added to the original supernatant. The combined supernatants were concentrated by vacuum dialysis to 24ml and stored at −20°C. One-dimensional 'rocket' immunoelectrophoresis was done with 2% antiserum in 0.056M-5,5-diethylbarbituric acid/0.031M-sodium barbitone/0.09M-glycine/0.048M-Tris base/0.04% calcium lactate/1% Triton X-100 in a 1% agarose (Miles type LE) gel. Two-dimensional immunoelectrophoresis was performed as described by Bjerrum (1977), but in the above buffer. Antibody titres were calculated from the gradients of curves of the precipitin area against

amount of antigen by using the method described by Harboe & Ingild (1976).

Preabsorption of antisera

Anti-(erythrocyte anion-transport protein) immunoglobulin G fraction or antiserum (which had been heated at 56°C for 30min) was preabsorbed with rabbit erythrocytes. Antibody (100μl) was added to 0.3ml of packed rabbit erythrocytes and incubated for 1h at 37°C. After centrifugation at 12000**g** for 20s in an Eppendorf microcentrifuge the supernatant was collected and the cells were washed with 0.2ml of 0.15M-NaCl and the washings added to the original supernatant. The combined supernatants were reabsorbed twice as described above by using two further batches of erythrocytes and the final supernatant was collected. This supernatant did not agglutinate rabbit erythrocytes.

Preabsorption of antibody preparations with crude rabbit erythrocyte lipid was done by incubating 100μl of antibody with 4.5mg of crude erythrocyte lipid (equivalent to lipid from 1ml of packed erythrocytes), which had been previously suspended by sonication in ice by using three 15s bursts at 6A (with a Dawes Soniprobe, type 1130) in 0.2ml of 0.15M-NaCl. After incubation at 37°C for 1h the mixture was centrifuged at 15000**g** for 2min in the Eppendorf centrifuge. The supernatant was removed, and reabsorbed twice, as described above, by using two further 4.5mg batches of lipid.

Staphylococcus aureus binding

S. aureus (Cowan 1 strain) cells were fixed and prepared as described by Kessler (1975) and stored as a 10% (w/v) suspension in phosphate-buffered saline containing 0.02% NaN_3 at 4°C. Before use the bacteria were washed twice with 0.15M-NaCl containing 5mM-EDTA, 0.05% Triton X-100 and 0.02% NaN_3. *S. aureus* binding to rabbit erythroid cells was done as described by Gahmberg *et al.* (1978). Samples (100μl aliquots) of a 5% (v/v) suspension of bone-marrow erythroid cells in phosphate-buffered saline (Denton & Arnstein, 1973) containing 1% bovine serum albumin and 0.02% NaN_3 (PBS/BSA medium) were mixed with various amounts of antiserum and incubated on ice for 30min. The cells were washed twice with 10ml of PBS/BSA medium. Next 50μl of a 10% suspension of fixed *S. aureus* was added and the cells were incubated at room temperature for 30min. The cells were washed with 3 × 10ml of PBS/BSA medium, smeared on glass slides and stained with the May–Grunwald/Giemsa stain (Dacie & Lewis, 1975).

Radioiodination of erythrocyte 'ghosts' and immunoprecipitation of anion-transport protein

Rabbit erythrocyte 'ghosts' were radiodinated by using lactoperoxidase as described by Boxer *et al.* (1974). The 'ghosts' were extracted with 4vol. of an ice-cold solution, pH9.6, containing 0.01M-glycine, 0.038M-Tris, 1% Triton X-100, 2% propan-2-ol and 2mM-phenylmethanesulphonyl fluoride (Bjerrum, 1977). After stirring for 20min, the mixture was centrifuged at 40000**g** for 1½h and the supernatant was frozen in liquid N_2, and stored at −70°C.

Samples of radioiodinated 'ghosts' solubilized in Triton X-100 as described above were incubated with anti-(rabbit anion-transport protein) antibody for 1h at 4°C. Then 100μl of a 10% suspension of fixed *S.* aureus was added and the incubation continued for a further 30min (Gahmberg *et al.*, 1978). The *S. aureus* cells were then washed three times with 0.15M-NaCl containing 5mM-EDTA, 0.5% Triton X-100 and 0.02% NaN_3. The bound protein was solubilized at 100°C for 3min in gel sample buffer (Fairbanks *et al.*, 1971) containing 5% 2-mercaptoethanol and 2mM-phenylmethanesulphonyl fluoride. Polyacrylamide-gel electrophoresis was done in the discontinuous buffer system of Laemmli (1971).

Serological methods

Sheep anti-(anion-transport protein) serum and human anti-(blood group I) serum were tested against erythrocytes of human blood-group I, human blood-group i and rabbit by standard direct agglutination methods (Boorman & Dodd, 1970). To test the ability of lipid preparations to inhibit agglutination, 3vol. of crude human erythrocyte lipid (7.8mg/ml) or crude rabbit erythrocyte lipid (6.8mg/ml), which had been previously sonicated in phosphate-buffered saline, was mixed with 1vol. of test serum or immunoglobulin G preparation incubated for 45min at room temperature, and the agglutination activity of the serum was tested as described above.

Results

The membranes of rabbit (New Zealand White) erythrocytes lack the periodate-staining sialic acid-rich glycoproteins found in the erythrocytes of most other species (Light & Tanner, 1977). The anion-transport protein is the only major integral membrane protein in these cells. The anion-transport was isolated from New Zealand White-rabbit erythrocyte membranes by extraction of the membranes with lithium 3',5'-di-iodosalicylate followed by solubilization in Nonidet P40 or Triton X-100. The preparation gave a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with the Coomassie Blue stain (Fig. 1*a*). The periodic acid/Schiff's-base carbohydrate stain showed a weak band corresponding to the anion-transport protein and material migrating with the marker-dye

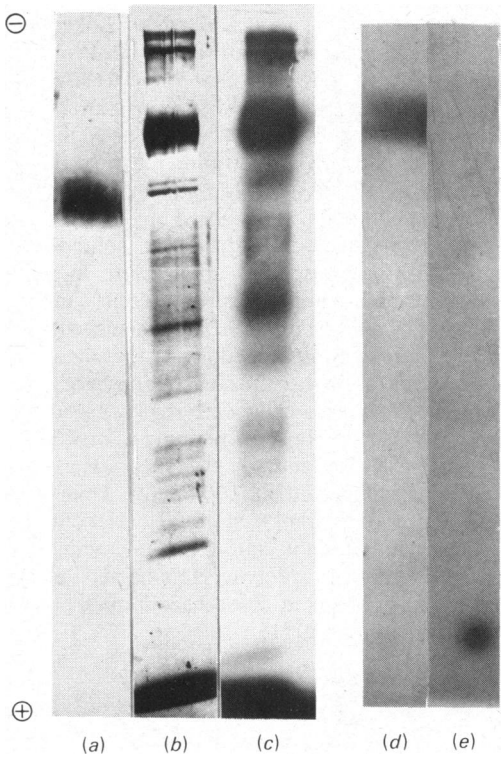


Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified anion-transport protein and immunoprecipitation of anion-transport protein*

(*a*) Rabbit erythrocyte anion-transport protein purified by extraction with Nonidet P40. For details see the Materials and methods section. (*b–e*) Immunoprecipitation of anion-transport protein: (*b*) Coomassie Blue stain and (*c*) radioautograph of radioiodinated rabbit erythrocyte 'ghosts' solublized in non-ionic detergent. The 'ghosts' were immunoprecipitated with (*d*) 2μl of anti-(anion-transport protein) serum or (*e*) 2μl of the preimmunization serum as described in the Materials and methods section, and radioautographs were prepared. Electrophoresis was in gels containing a concentration gradient of acrylamide (5–15%, w/v) with an overlay containing 5% (w/v) acrylamide, in the buffer system of Laemmli (1970).

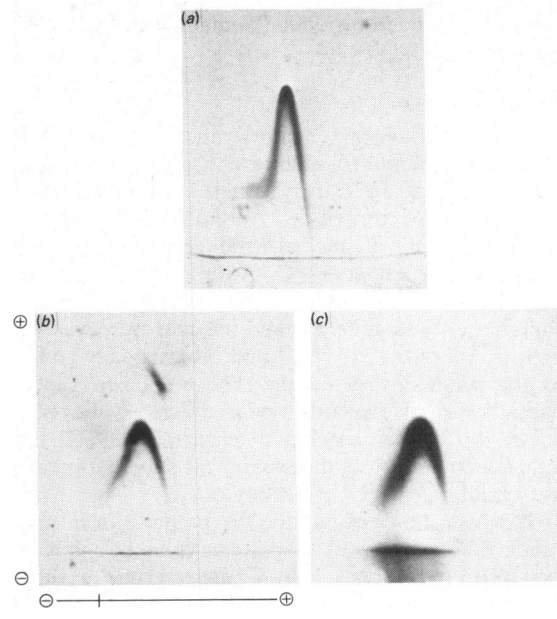


Fig. 2. *Two-dimensional crossed immunoelectrophoresis with anti-(anion-transport protein) serum A*

The second-dimension gel in each case contained 2% anti-(anion-transport protein) serum A. (*a*) Electrophoresis of 4μg of rabbit anion-transport protein purified by Nonidet P40 extraction; Coomassie Blue stain. (*b*, *c*) Electrophoresis of radioiodinated rabbit erythrocyte 'ghosts' that had been solubilized in non-ionic detergent (the same sample as that shown in Figs. 1*b* and 1*c*): (*b*) Coomassie Blue stain. The mark above the precipitin arc is a staining artefact. (*c*) radioautograph.

front (probably glycolipids). Faintly staining bands with a mobility faster than that of the anion-transport protein were also present. Solubilization of the anion-transport protein was equally effective with either Triton X-100 or Nonidet P40, but Nonidet P40 was routinely used because preparations in this detergent appeared to be less prone to aggregation after freezing and storage at −70°C.

The anion-transport-protein preparation was used to immunize a sheep to prepare an anti-(anion-transport protein) antiserum. Two-dimensional crossed immunoelectrophoresis (in the presence of Triton X-100) of the antiserum against the anion-transport protein preparation gave a strong precipitin line (Fig. 2*a*).

When rabbit erythrocyte 'ghosts' that had been radioiodinated with lactoperoxidase (Figs. 1*b* and 1*c*) were run against the antiserum on two-dimensional crossed immunoelectrophoresis, a single major precipitin line was obtained that stained with Coomassie Blue and contained the radioactive label (Figs. 2*b* and 2*c*). A broad smear of radioactivity was found in the first dimension (which did not contain antibody), and a sharp radioactive band was observed at the interface of the first dimension and the antibody-containing gel. This radioactivity did not have the appearance of a precipitin line and was probably aggregated protein that had not migrated out of the first dimension and that had collected at the interface of the two gels. The electrophoretic mobility of this material in the first dimension

suggests that it was probably the anion-transport protein, which has a tendency to aggregate in non-ionic-detergent solutions.

When the same radioactive erythrocyte 'ghost' preparation was immunoprecipitated with the antibody and fixed *S. aureus* and the labelled protein in the precipitate was separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Figs. 1*d* and 1*e*), the labelled anion-transport protein was found to be immunoprecipitated by the antiserum. A parallel experiment with the preimmunization serum from the same animal did not result in the precipitation of the labelled anion-transport protein.

The antiserum had an agglutination titre of 1:512 against rabbit erythrocytes, whereas the preimmunization serum from the same sheep gave a titre of 1:16 under the same conditions. An immunoglobulin G fraction from the antiserum did not agglutinate human blood-group-I erythrocytes at 37°C, but at 11°C it agglutinated these erythrocytes with a titre of 1:4, human blood-group-i erythrocytes with a titre of 1:8, and rabbit erythrocytes at a titre of 1:64. Although in human erythrocytes the blood-group-I antigen is known to be carried on both the anion-transport protein and glycolipids (Fukuda *et al.*, 1979), these results suggest that the antiserum did not contain anti-(blood-group-I) antigen activity. Preabsorbtion of the antiserum with a crude lipid fraction prepared from rabbit erythrocytes did not change the agglutination titre of the antiserum against rabbit erythrocytes. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of this lipid fraction gave no protein-staining bands, but gave a strong periodic acid/Schiff-staining band that migrated with the marker front. This band probably represented membrane glycolipids. The lipid preparation decreased the agglutination of human blood-group-I erythrocytes by anti-(blood-group-I) antiserum from a titre of 1:64 to 1:8, and a similar crude lipid preparation from human erythrocytes gave the same degree of inhibition. Since a proportion of the blood-group-I antigen is known to be bound to glycolipids, these results confirm that the crude lipid preparation contained glycolipids. Quantitative one-dimensional 'rocket' immunoelectrophoresis of the anion-transport protein against the antiserum, and against the antiserum that had been preabsorbed with the crude lipid fraction, was performed (Fig. 3). Calculations based on the area under the two groups of precipitin 'rockets' showed that the antiserum that had been preabsorbed with crude lipid had $92 \pm 9\%$ (S.D.; $n = 3$) of the titre against the anion-transport protein of the unabsorbed serum. These results suggest that the antiserum contained no significant activity against membrane glycolipids and that the immunoprecipitates observed on 'rocket' immunoelectrophoresis are not the result of the reaction of glycolipids tightly bound to the anion-transport protein with anti-glycolipid antibodies in the antiserum.

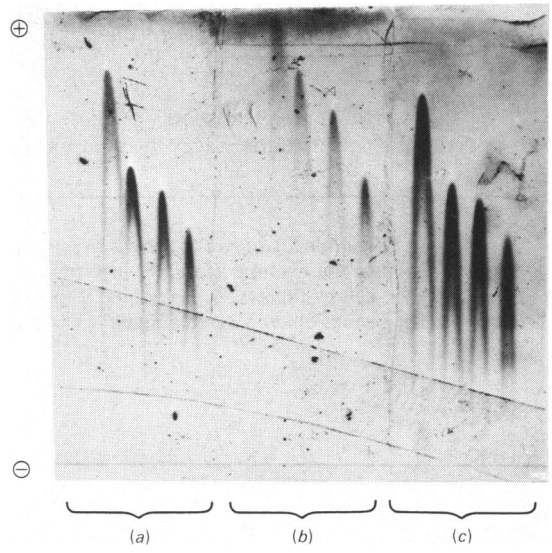


Fig. 3. *One-dimensional 'rocket' immunoelectrophoresis of absorbed anit-(anion-transport protein) preparations*
The antiserum preparations were run at the same time on different portions of the same immunoelectrophoresis plate. Each gel contained a concentration of antiserum equivalent to 2% of the original untreated antiserum. Each antiserum was run against 5μg, 2.5μg, 1μg and 0.5μg (from left to right) of purified anion-transport protein. (*a*) Anti-(anion-transport protein) serum preabsorbed with crude rabbit erythrocyte lipid; (*b*) anti-(anion-transport protein) serum preabsorbed with rabbit erythrocytes: (*c*) untreated anti-(anion-transport protein) serum. All gels are stained with Coomassie Blue. Other details are given in the Materials and methods section.

A similar method was used to determine whether the antibody reacted with both the extracellular and cytoplasmic domains of the anion-transport protein. The antiserum was absorbed with rabbit erythrocytes until no agglutinating activity against rabbit erythrocytes was detectable. Quantitative 'rocket' immunoelectrophoresis of the anion-transport protein preparation against the unabsorbed serum and the serum preabsorbed with rabbit erythrocytes (Fig. 3) showed that the preabsorbed serum had 64 and 71% of the titre of the unabsorbed serium in two different experiments. Thus the antiserum reacts with both the cytoplasmic and extracellular domains of the protein, with 65–70% of the activity directed against determinants in the cytoplasmic or intramembranous domains of the protein.

Detection of the anion-transport protein at the surface of erythroid cells

The sheep anti-(anion-transport protein) antiserum was used to define the stages in erythroid-cell development at which the anion-transport protein becomes expressed at the cell surface, by using the method described by Gahmberg *et al.* (1978). Rabbit bone-marrow erythroid cells were incubated with the anti-(anion-transport protein) antiserum

Table 1. *Relative binding of S. aureus to antibody-treated bone-marrow erythroid cells from normal rabbits*
The Table shows the average score per erythroid cell. Score points were given for the number of *S. aureus* cells bound to each cell as follows: 0, no points; 1–3, 1 point; 4–6, 4 points; 7–9, 7 points; 10 or more, 10 points. At least 30 cells of each cell type were counted.

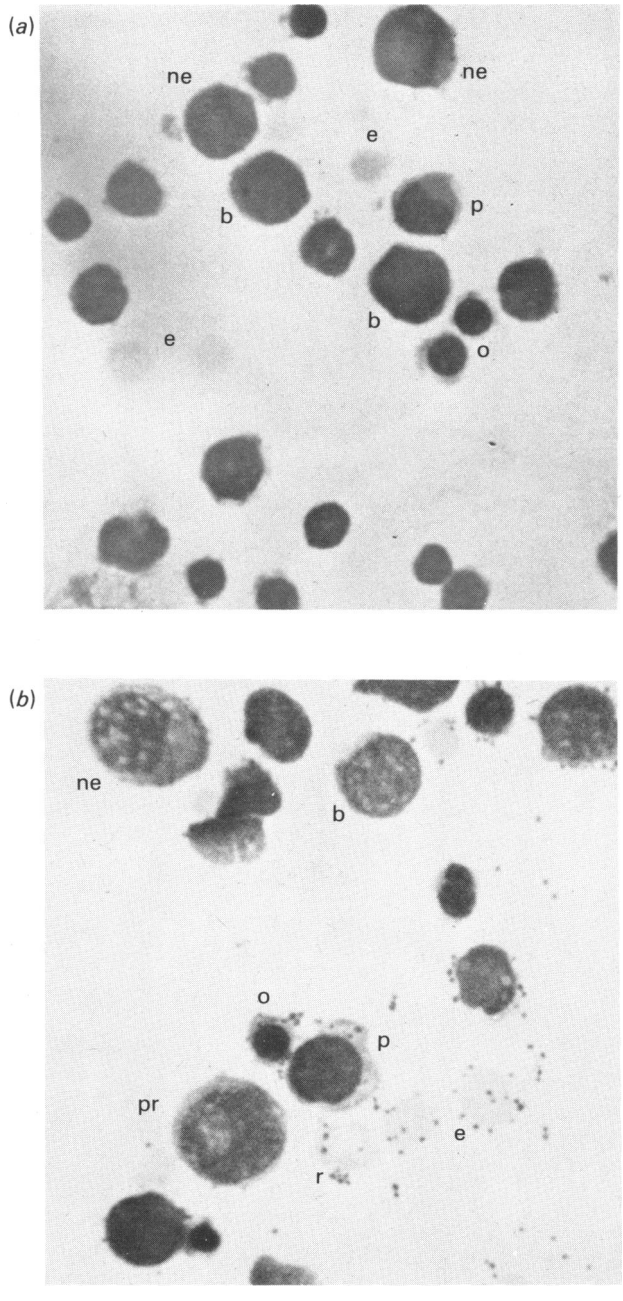
		Average score/cell					
		Erythrocyte-absorbed			Lipid-absorbed		
Cell type	Amount of serum (μl) ...	0.125	0.375	1.0	0.125	0.375	1.0
Pronormoblast		0.13	0.0	0.13	1.3	1.8	2.4
Basophilic normoblast		0.125	0.07	0.07	1.1	2.7	4.4
Polychromatic normoblast		0.13	0.12	0.03	1.9	3.7	4.9
Orthochromatic normoblast		0.09	0.03	0.05	3.1	5.1	6.2
Reticulocyte		0.08	0.07	0.09	4.6	6.5	8.6
Erythrocyte		0.09	0.02	0.12	4.1	7.0	9.1
Non-erythroid		0.28	0.24	0.47	1.9	1.8	1.4
Total no. of cells counted		521	347	503	480	533	481

Table 2. *Relative binding of S. aureus to antibody-treated bone-marrow erythroid cells from bled rabbits*
Details are given in the legend to Table 1.

		Average score/cell					
		Erythrocyte-absorbed			Lipid-absorbed		
Cell type	Amount of serum (μl) ...	0.125	0.375	1.0	0.125	0.375	1.0
Pronormoblast		0.20	0.16	0.20	0.34	0.45	1.8
Basophilic normoblast		0.19	0.10	0.25	0.81	0.7	2.2
Polychromatic normoblast		0.13	0.03	0.23	0.42	0.9	3.5
Orthochromatic normoblast		0.19	0.15	0.14	0.42	2.0	4.6
Reticulocyte		0.56	0.12	0.22	1.6	5.8	5.9
Erythrocyte		0.13	0.35	0.17	1.7	5.4	6.8
Non-erythroid		0.24	0.06	0.18	0.7	0.4	1.3
Total no. of cells counted		258	249	252	267	282	279

Table 3. *Relative binding of S. aureus to antibody-treated bone-marrow erythroid cells from phenylhydrazine-treated rabbits*
Details are given in the legend to Table 1.

		Average score/cell					
		Erythrocyte-absorbed			Lipid-absorbed		
Cell type	Amount of serum (μl) ...	0.125	0.375	1.0	0.125	0.375	1.0
Pronormoblast		0.03	0.03	0.0	2.0	1.4	0.6
Basophilic normoblast		0.03	0.18	0.1	2.6	3.0	1.6
Polychromatic normoblast		0.11	0.11	0.29	5.3	3.4	2.0
Orthochromatic normoblast		0.04	0.27	0.35	5.5	3.6	3.3
Reticulocyte		0.04	0.09	0.17	9.0	7.6	3.7
Erythrocyte		0.1	0.02	0.28	9.7	8.6	4.1
Non-erythroid		0.0	0.0	0.11	1.8	1.1	0.73
Total no. of cells counted		298	464	368	320	344	388



EXPLANATION OF PLATE 1

Binding of anti-(anion-transport protein) antibody to erythroid cells observed by the S. aureus 'rosette' technique
Bone-marrow erythroid cells from a rabbit made anaemic by bleeding were treated with (*a*) 1 μl of antibody that had been preabsorbed with rabbit erythrocytes or (*b*) 1 μl of antibody that had been preabsorbed with crude erythrocyte lipid, followed by fixed *S. aureus*. Photomicrographs (magnification X1000) of May–Grunwald/Giemsa-stained smears of the preparations are shown. Abbreviations used: pr, pronormoblast; b, basophilic normoblast; p, polychromatic normoblast; o, orthochromatic normoblast; r, reticulocyte; e, erythrocyte; ne, non-erythroid cell. The identification of the cells was confirmed from the original colour transparencies of the photomicrographs.

(serum A), and the antibody that bound to the cell surface was detected by the subsequent binding of fixed *S. aureus*. After incubation with the bacteria the cells were smeared and stained to distinguish the different cell stages. Counts were made of the cell types and number of bacteria bound to each cell type. The antiserum used was preabsorbed with the crude rabbit lipid preparation. Control experiments used the same serum that had been preabsorbed with rabbit erythrocytes to determine the extent of non-specific binding of the bacteria. Plate 1 shows typical fields of erythroid cells incubated with the anti-(anion-transport protein) serum and the same serum that had been preabsorbed with erythrocytes.

To obtain quantitative estimates of the number of bacteria bound to each cell type, a scoring system was used (see Table 1). This simplified the counting of the bacteria bound to each cell type, and also allowed for the fact that there was some variation in the number of bacteria bound to a given cell type. This is to be expected, since cell maturation is a continuous process, and a given histochemically defined class of cells contains cells that are entering and leaving their class, giving rise to some variation in their properties. With reticulocytes and erythrocytes, which often bound very large numbers of bacteria, it became difficult to count accurately the number of bacteria bound. The cells were grouped into those binding 0, 1–3, 4–6, 7–9 and 10 or more bacteria, and were given scores of 0, 1, 4, 7 and 10 respectively. The mean score for each cell type was determined by averaging the scores obtained on counting 30 or more cells of each type.

When the experiment was performed with bone-marrow erythroid cells from a normal rabbit, the results shown in Table 1 were obtained. Although the pronormoblasts bound small numbers of bacteria (similar to the numbers of bacteria bound to non-erythroid cells), there was a progressive increase in the number of bacteria bound with increasing maturity of the cells up to the reticulocyte stage. Erythrocytes bound slightly more bacteria than did reticulocytes. The absolute number of bacteria bound to the cells depended on the concentration of the antiserum used. When the antiserum was preabsorbed with rabbit erythrocytes, very little binding of bacteria to bone-marrow cells was observed. Similar results were obtained with erythroid cells from rabbits made anaemic by bleeding (Table 2) and from rabbits made anaemic by phenylhydrazine treatment (Table 3). These experiments were repeated by using an immunoglobulin G fraction of the sheep anti-(rabbit anion-transport protein) serum obtained from a later bleeding of the same sheep (serum B) and gave results similar to those above. Since the number of bacteria bound was dependent on the relative concentration of cells and antibody, the scores for each cell type were normalized against the average number of bacteria bound to the erythrocytes in the same experiment to facilitate comparison. The averaged results for three experiments are shown in Fig. 4.

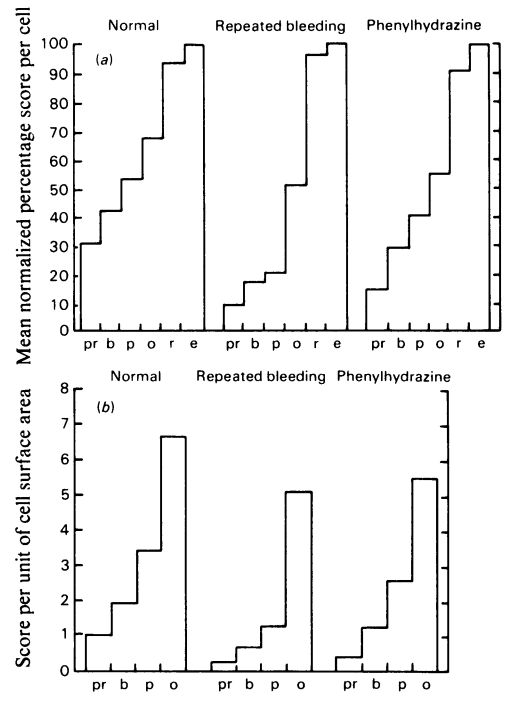


Fig. 4. *Binding of S. aureus to anti-(anion-transport protein)-antibody-treated bone-marrow erythroid cells*
(*a*) Each result is the mean from three experiments, and the histograms show the results from normal bone marrow ('Normal'), and from bone marrow from animals made anaemic by bleeding ('Repeated bleeding') and by phenylhydrazine treatment ('Phenylhydrazine'). Within each histogram the bars show (from left to right) the binding to pronormoblasts (pr), basophilic normoblasts (b), polychromatic normoblasts (p), orthochromatic normoblasts (o), reticulocytes (r) and erythrocytes (e). The mean normalized percentage score per cell is the total score of bacteria bound per cell to a given cell type expressed as a percentage of the total score of bacteria bound per erythrocyte in the same experiment. (*b*) The results in (*a*) for the stages earlier than the orthochromatic normoblast have been corrected for the change in surface area of the cells (relative to the orthochromatic normoblast; see the text). The score per unit of cell surface area represents the mean normalized percentage score of bacteria bound, corrected for the change in cell surface area, for each cell type (arbitrary units). Other details are as described for (*a*).

Discussion

The sheep antiserum against the rabbit anion-transport protein was reactive with antigenic determinants that are present in both the extracellular and cytoplasmic or intramembranous domains of this membrane-penetrating protein. The reactivity with the extracellular domain of the protein was not an artefact resulting from the association of tightly bound glycolipids with the protein and the presence of anti-glycolipid antibodies in the antiserum. Other workers have prepared antibodies against the human anion-transport protein, but these have been shown to react only with determinants in the *N*-terminal cytoplasmic domain of the protein (England & Steck, 1978; Fukuda *et al.*, 1978).

We have used the antibody to examine the surface of erythrocyte precursor cells in the bone marrow for the presence of the anion-transport protein by using the *S. aureus* 'rosette' technique. Tables 1–3 show that there was a progressive increase in the number of bacteria bound with increasing maturity of the cells until the reticulocyte stage is reached. There was little difference in the number of bacteria bound to the reticulocyte and erythrocyte stages.

Although the exact relationship between the number of bacteria bound and the surface concentration of immunoglobulin bound to the cell is not known, it seems reasonable to suppose that an increase in the number of bacteria bound reflects an increase in the surface concentration of bound immunoglobulin and therefore of the concentration of anion-transport protein on the cell surface. Other workers who have used this technique have made the same assumption (Gahmberg *et al.*, 1978).

The results in Tables 1–3 and Figs. 4(*a*), 4(*c*) and 4(*e*) are expressed as the score of bacteria bound per cell for each cell type. However, there is a progressive decrease in the size and surface area of erythroid cells during maturation. From average diameters of 18 μm, 15 μm, 12.5 μm, 10 μm, 9 μm and 7 μm for the pronormoblast, basophilic normoblast, polychromatic normoblast, orthochromatic normoblast, reticulocyte and erythrocyte respectively (Yataganas *et al.*, 1970), the relative surface areas of these cells can be calculated to be in the ratios 1:0.69:0.48:0.31:0.25:0.15. The decrease in surface area between the orthochromatic-normoblast, reticulocyte and erythrocyte stages results from membrane loss, so that in these cases the change in the score of bacteria bound per cell rather than the bacteria bound per unit surface area of the cell reflects the real change in the total amount of anion-transport protein in the plasma membrane; however, the successive maturation stages up to the orthochromatic normoblast result from cell division. Although this produces a decrease in diameter of the daughter cells, the total plasma-membrane area of the daughter cells increase during this process. In these early cells, the score of bacteria bound per unit area of cell surface gives a more proper basis for comparison of the relative concentrations of the anion-transport protein. When the results are expressed in this way (Figs. 4*b*, 4*d* and 4*f*), the increase in concentration of the anion-transport protein as the cells mature is more striking.

Comparison of the results for normal bone marrow with those for bone marrow from animals made anaemic by bleeding or phenylhydrazine treatment suggest that during bone-marrow stress the incorporation of the anion-transport protein is delayed towards the later cell stages. This may reflect the increasing rate of cell maturation under marrow stress. Under these conditions, larger reticulocytes tend to be formed as a result of enucleation at the polychromatic-normoblast stage, with the omission of the cell division leading to the orthochromatic normoblast (Brecher & Stohlman, 1961; Borsook *et al.*, 1962). These reticulocytes are more active in haemoglobin synthesis than are normal reticulocytes (Borsook *et al.*, 1962). Hillman & Giblett (1965) have also shown that, in man, bone-marrow stress results in the appearance of the blood-group-i antigen (the incomplete form of the normal adult blood-group-I antigen) in the circulatory erythrocytes.

In a previous paper (Light and Tanner, 1977), we detected small amounts of the anion-transport protein in rabbit bone-marrow erythroid cells, but attributed this to contamination from reticulocytes and erythrocytes in the incompletely purified nucleated erythroid-cell preparations. The present results show that the anion-transport protein is a component of nucleated erythroid cells. More recently, Fukuda *et al.* (1980) have reported the immunoprecipitation of small amounts of the anion-transport protein from human erythroblasts derived from erythroid burst colonies in culture. However, those authors did not state the apparent maturation stage of the erythroblasts. Our results suggest that most of the anion-transport protein present in the mature erythrocyte is inserted into the plasma membrane between the polychromatic-normoblast and reticulocyte stages. This is consistent with studies on membrane protein synthesis in reticulocytes from anaemic rabbits, which showed the anion-transport protein was only incorporated into the membrane of the most immature (Type I) reticulocyte (Light & Tanner, 1978). The low incorporation observed in these cells suggests that the shutdown in the synthesis of this major membrane protein has been initiated before the cells enter the circulation.

We thank Dr. I. Fraser for his advice on the identification of bone-marrow cells, Dr. J. Bourne for

performing the immunization of the sheep, and Dr. P. Ball and Dr. A. Docherty for gifts of *S. aureus* (Cowan I). B. M. J. F. was the recipient of a Science Research Council Studentship. The work was supported in part by a grant from the Medical Research Council.

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