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Research Article

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Abnormal Erythrocyte Membrane Protein Pattern in Severe Megaloblastic Anemia

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A B S T R A C T The erythrocyte membrane protein pattern of patients with megaloblastic anemia was determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In severe megaloblastic anemia, secondary either to folic acid or vitamin B_{12} deficiency, the erythrocyte membrane protein pattern was grossly abnormal, lacking bands 1, 2 (spectrin), and 3 and having several diffuse, faster migrating bands. After adequate vitamin replacement therapy, the erythrocyte membrane protein pattern returned to normal. In mild megaloblastic anemia, secondary either to folic acid or vitamin B_{12} deficiency, and in severe iron deficiency anemia, the erythrocyte membrane protein pattern was normal. Erythrocyte membrane protein pattern of normal membranes did not change after mixing with abnormal membranes before polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Protease activity extracted from membranes of megalocytes was not different from normal. These findings indicate that the erythrocyte membrane protein pattern is abnormal in severe megaloblastic anemia and that this abnormality is not secondary to increased activity of the endogenous erythrocyte membrane proteinase.

INTRODUCTION

The biochemical lesion in megaloblastic anemia is characterized by a rate of DNA replication slower than normal (1), whereas RNA and protein synthesis in megaloblastic erythroblasts is unaffected (2). The erythrocytes in this disease are macroovalocytic, are less filtrable than normal erythrocytes (3), and have a reduced life span (4, 5). Although macrocytosis usually is related to the presence of fewer divisions of nucleated precursors in megaloblastic anemia, the pathophysiology of the acquired cell membrane abnormality is undefined. In this communication the erythrocyte membrane protein pattern in severe megaloblastic anemia, secondary to folic acid or vitamin B_{12} deficiency, has been investigated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (PAGE-SDS).[1]

METHODS

Blood was obtained from patients with proven megaloblastic anemia or iron deficiency anemia. Laboratory personnel served as controls. Blood was collected in heparin and used within 1–2 h of collection. Routine hematological data were obtained by standard laboratory methods. Serum folate levels were measured by the microbiologic assay technique of Waters and Mollin (6). Serum vitamin B_{12} levels were measured by the radioisotopic method of Lau and associates (7). The diagnosis of pernicious anemia was proven by Schilling tests. Hemoglobin-free erythrocyte membranes were isolated at 0°C by the method of Dodge et al. (8), as modified by Fairbanks et al. (9), and processed immediately or stored at −70°C. Care was taken to remove the buffy coat after each wash of the erythrocytes, to aspirate the pellet of unlysed leukocytes after each wash of the membranes, to keep the membranes at 0°C, and to work as rapidly as possible to minimize proteolysis

[1] *Abbreviation used in this paper:* PAGE-SDS, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

(9). In some experiments leukocytes were removed from fresh heparinized blood by filtration through Whatman no. 2 filter paper (Whatman, Inc., Clifton, N. J.). The filtrate was then layered over 8.25% Ficoll (Pharmacia, Uppsala, Sweden), specific gravity 1.077, and centrifuged at 1,000 g for 20 min (10). Erythrocytes packed in the Ficoll layer were virtually free of contaminating leukocytes. Erythrocytes were then washed and membranes prepared as described above. PAGE-SDS was performed as previously described (9) and the gels were stained with Coomassie Brilliant Blue. The bands were numbered according to the system instituted by Fairbanks et al. (9) and extended by Steck (11). The proportion of various bands was assessed by densitometry of the stained gels with a Gilford spectrophotometer and model 2410 linear transport accessory (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), scanning through a 0.1-mm slit at 620 nm.

Mixing experiments were performed by incubating equal amounts (wt/wt) of megaloblastic and normal erythrocyte membranes for 1–3 h at 37°C in a metabolic shaker before PAGE-SDS in the presence or absence of Trasylol (Sigma Chemical Co., St. Louis, Mo.), final concentration 1 or 10 kallikrein inactivator U/ml, and benzamidine, final concentration 2 or 20 mM (12). Protease activity of normal and abnormal erythrocyte membranes was extracted in 1 M potassium thiocyanate solution and assessed by the method of Morrison and Neurath (13), as modified by Moore et al. (14), with ^{14}C-labeled hemoglobin as substrate. The latter was prepared by incubating reticulocyte-rich blood obtained from patients with hemolysis with ^{14}C-amino acid mixture (uniformly labeled, New England Nuclear, Boston, Mass.) for 2 h. ^{14}C-hemoglobin was then isolated, converted to the carbon-monoxy form (15), and purified as described by Winter-halter and Huehns (16). The assay mixture consisted of 20 mg ^{14}C-labeled hemoglobin substrate, 0.8–1.6 mg enzyme extract, and enough potassium phosphate buffer, pH 7.4, to

bring the final volume to 7 ml (14). Each compared set of assay mixtures contained the same amount of normal or megaloblastic enzyme extract as determined by the method of Lowry et al. (17). The test mixtures were then incubated at 37°C for 24 h in a metabolic shaker. Aliquots of 2 ml were then precipitated by addition of an equal volume of 10% TCA, centrifuged, and the radioactivity determined in the supernate. ^{14}C-radioactivity was determined in a Packard liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.) with an efficiency of more than 95%. Erythrocyte membrane protein was measured by the method of Lowry et al. (17). The statistical significance was determined by the Student's or paired t test whenever applicable (18).

RESULTS

Pertinent clinical and hematological data are summarized in Table I. The first five patients had moderate-to-severe anemia with pronounced megaloblastic marrow changes secondary to folic acid or vitamin B_{12} deficiency. All these patients had a grossly abnormal erythrocyte membrane protein pattern as determined by PAGE-SDS. PAGE-SDS of the erythrocyte membrane proteins of each patient was performed at least on three separate occasions and each time in triplicate. A typical densitometric scan of the SDS-PAGE of the erythrocyte membranes of these patients is shown in Fig. 1. The patient's erythrocyte membranes, when compared with the control membranes, show deficiency of bands 1, 2 (spectrin), and 3 and the appearance of several abnormal faster migrating diffuse

TABLE I
Summary of Clinical, Hematological, and Membrane Data

Patient	Age/Sex	Hemoglobin	Mean corpuscular volume	Serum folate	Serum B_{12}	Marrow megalo-blastosis	Cause of deficiency	Erythrocyte membrane protein
	yr	*g/dl*	*fl*	*ng/ml*	*pg/ml*			
1. A. M.	59/F	8.7	104	<2.0	239	Severe	Dilantin	Abnormal
2. L. R.	40/M	6.9	103	<2.0	300	Severe	Alcoholism	Abnormal
3. E. D.	34/F	3.5	144	<2.0	436	Severe	Alcoholism	Abnormal
4. R. W.	52/M	8.5	130	<2.0	740	Severe	Alcoholism	Abnormal
5. G. B.	51/F	7.1	101	9.4	70	Severe	Pernicious anemia	Abnormal
6. M. M.	36/F	11.6	109	13.0	63	Not done	Pernicious anemia	Normal
7. M. O.	88/F	12.0	120	6.0	122	None	Pernicious anemia	Normal
8. J. M.	18/F	10.8	94	2.0	279	Minimal	Starvation	Normal
9. J. A.	62/M	13.0	101	2.0	320	Minimal	Alcoholism	Normal
10. A. W.*	23/M	5.1	70	7.0	380	None	Blood loss	Normal
11. R. B.*	75/F	5.0	72	5.6	762	None	Blood loss	Normal
12. D. B.*	25/M	5.9	61	—	—	None	Blood loss	Normal
Controls		14.6±1.3	86±6	>2.6	200–900	None	—	Normal‡

* Has proven iron deficiency anemia secondary to chronic blood loss.
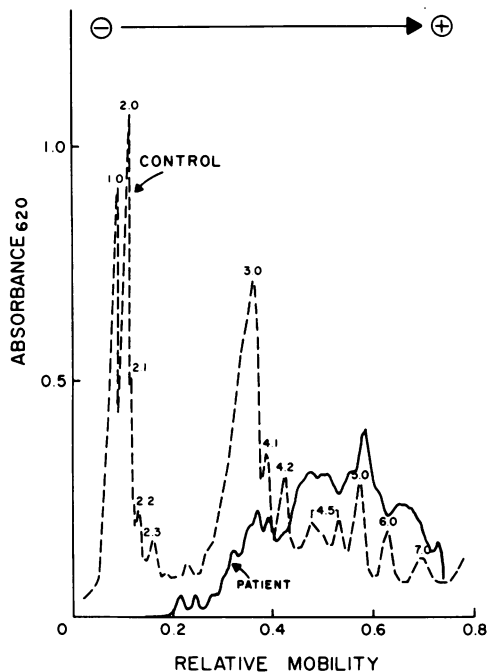‡ 40 determinations, each in triplicate.

FIGURE 1 Spectrophotometric scan of electrophorograms of erythrocyte membrane proteins. Equal amounts of protein (35 μg) were applied to each gel. Gels were stained with Coomassie Blue and scanned at 620 nm. In normal membranes the expected seven major bands were present. In megaloblastic anemia the pattern is abnormal, lacks bands 1, 2, and 3, and has several diffuse fast moving bands.
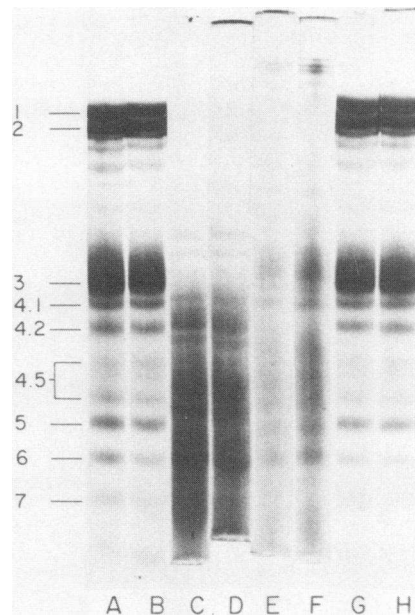
FIGURE 2 SDS-polyacrylamide (5%) gel electrophoresis of erythrocyte membrane proteins. About 35 μg of protein was applied to each gel. Gels were stained with Coomassie Blue. A & B, normal control erythrocyte membrane proteins. C & D, membrane protein pattern of patient A. M. with severe megaloblastic anemia secondary to folic acid deficiency. E & F, membrane protein pattern of the same patient 4 wk after vitamin replacement therapy. There is a faint reappearance of bands 1, 2, and 3. G & H, membrane protein pattern 6 mo after therapy.

bands. The abnormal pattern shown in Fig. 1 was always reproducible.

Fig. 2 is a photograph of serial PAGE-SDS gels of patient A. M. Membranes C and D are grossly abnormal as described for Fig. 1. After 4 wk of folic acid replacement therapy, membranes E and F show faint reappearance of bands 1, 2 (spectrin), and 3. Membranes G and H, isolated 6 mo after therapy, are normal. At this time the serum folic acid and mean corpuscular volume were normal and the bone marrow showed a normoblastic maturation pattern. The hemoglobin did not rise above 10.2 g/dl because of a coexistent renal failure.

PAGE-SDS gels of patients nos. 2–5, identified in Table I, also show a grossly abnormal membrane protein pattern as described above. Four months after folic acid therapy, when serum folic acid, hemoglobin concentration, and mean corpuscular volume were all within normal limits, patients L. R. and E. R. had normal membrane protein patterns. Bone marrow aspiration of patient no. 2 at this time showed no megaloblastic changes. Patients nos. 6–9, with mild anemia and minimal or no megaloblastic marrow changes, had normal membranes. That the abnormal membrane protein pattern is not a non-

specific consequence of severe anemia is demonstrated by the normal membrane protein pattern of patients nos. 10–12 with iron deficiency anemia of comparable severity.

Fig. 3 demonstrates the results of mixing experiments. Membranes A are normal control membranes; membranes B are those of patient G. B. and are grossly abnormal; membranes C contain equal amounts of normal and abnormal membranes that were incubated at 37°C for up to 3 h before PAGE-SDS. It is obvious that the abnormal membranes did not cause proteolysis of the normal ones. The membrane protein pattern shown in C gels (Fig. 3) was the same whether the mixing experiments included the presence of the proteolytic inhibitors trasylol or benzamidine or not and whether their incubation period before PAGE-SDS was 1, 2, or 3 h. Mixing experiments were performed with membranes from patients E. D., R. W., and G. B. with similar results.

Table II shows no difference between the protease activity of enzyme extracts from normal and megalocytic membranes. When the ^{14}C-hemoglobin substrate was incubated in a final volume of 7 ml of buffer
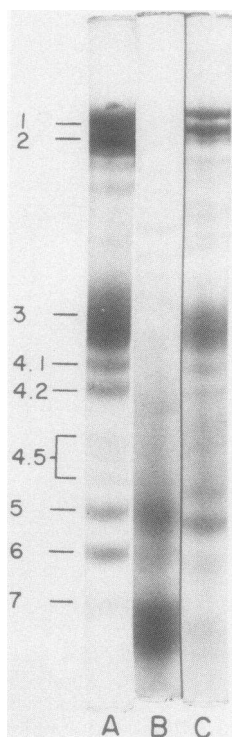
FIGURE 3 SDS-polyacrylamide (5%) gel electrophoresis of erythrocyte membrane proteins. Gels were stained with Coomassie Blue. A, normal control erythrocyte membrane proteins containing 36 μg. B, membrane proteins (34 μg) of patient G. B. C, membrane protein pattern of a mixture of 15 μg of A membranes and 15 μg of B membranes incubated at 37°C for 3 h before PAGE-SDS.

in the absence of any enzyme extract, radioactivity released into the supernate was less than 10% of the corresponding control values.

Total erythrocyte membrane protein content of megalocytes was 0.72±0.14 mg/10⁹ cells (nine determinations) which is significantly different ($P < 0.001$) from the normal 0.43±0.04 mg/10⁹ cells (10 deter-

TABLE II
Results of the Proteolytic Activity of 1 M Potassium Thiocyanate Extracts of Erythrocyte Membranes

| | Experiment no. | Activity | | Percent control value |
		Patient	Control	
		cpm/ml		%
Patient R. W.	1	2,812	3,232	87
	2	3,192	3,099	103
Patient G. B.	1	8,256	8,339	99
	2	7,985	8,405	105
	3	9,344	9,834	94

minations). Total erythrocyte membrane protein content of microcytic erythrocytes from patients with iron deficiency anemia, in contrast, was 0.24±0.07 (five determinations) which is also significantly different from the normal value ($P < 0.001$).

DISCUSSION

The present study was designed to find out if erythrocyte membrane protein abnormalities in megaloblastic anemia would explain the decreased filtrability of megalocytes previously reported (3). The results indicate that erythrocyte membrane protein patterns are indeed grossly abnormal in severe megaloblastic anemia with absence of bands 1, 2 (spectrin), and 3 and the presence of several abnormal faster migrating diffuse bands (Fig. 1–3). These abnormalities, like the decreased filtrability of megalocytes (3), are related to the severity of the megaloblastic anemia and not to the increase in the mean corpuscular volume of erythrocytes (Table I).

The altered membrane protein pattern could be the result of decreased production, increased proteolysis, or instability of the absent bands. Decreased membrane protein production seems unlikely in megaloblastic anemia in view of the fact that RNA and protein synthesis are relatively unaffected (2) in this disorder. Moreover, total erythrocyte membrane protein content of megalocytes was significantly higher than normal membranes. Increased proteolysis of the absent bands is also unlikely in view of the finding that mixing experiments did not result in an alteration of the normal membrane protein pattern. Furthermore, direct assessment of the protease activity extracted from megaloblastic erythrocyte membranes was not different from normal. These findings rule out the possibility of contamination of erythrocyte membranes with leukocyte lysosomal enzymes and of increased activity of the endogenous erythrocyte membrane proteinase activity described by Morrison and Neurath (13). The third possible explanation for these findings is instability of the high molecular weight components of the erythrocyte membrane proteins in severe megaloblastic anemia. The pathophysiology of this abnormality, and whether it is an in vivo or in vitro phenomenon, remains to be determined.

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