Evidence for erythrocyte membrane glycoproteins being carriers of blood-group P_1 determinants

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The contribution of different membrane constituents to the bloodgroup P_1 activity of human erythrocytes was investigated. Pronase digestion of native red cell stroma or partition between butanol and water had no serologically detectable effect, whereas pronase-treatment of previously butanol-extracted membranes liberated virtually all blood-group P_1 determinants from the ghosts. On Laemmli gels, all P_1 activity was found in the band 4.5 region. Thus it is concluded that, in addition to the well-documented P_1 glycolipid, also membrane glycoproteins are carriers of blood-group P_1 determinants.

Blood-group P *Blood-group substances* *Erythrocyte membrane* *Glycoprotein* *Glycolipid*

1. INTRODUCTION

The blood-group P system comprises 5 phenotypes, P_1, P_2, P_1^k, P_2^k and p, which are determined by 3 different antigens, P^k, P and P_1. The group P substances of human erythrocytes have been described as being the lipids globotriaosyl ceramide (P^k) [1], globoside (P) [1] and the ceramide pentasaccharide [2]:

Gal α1–4 Gal β1–4 GlcNAc β1–3 Gal β1–4 Glc-ceramide (P_1)

Whilst globoside and the ceramide trihexoside are common constituents of the erythrocyte membrane occurring in high concentrations (4.2 and 16.7 μmol/100 ml packed P_1 cells, respectively [3]), the P_1 active glycosphingolipid was detected only in minute amounts [2]. A significant contribution of this P_1-active glycolipid to the blood-group characteristics of the red cell seemed therefore at least worth questioning. As investigations on the blood-group systems ABH [5–7] and Ii [4] also had revealed that the predominant number of the respective antigens is part of the 'band 3' glycoprotein, the major intrinsic membrane protein of the erythrocyte, experiments were performed to examine the glycoprotein material of the red blood cell for the occurrence of P_1 antigenic sites.

2. MATERIALS AND METHODS

Rabbit anti-P_1 antiserum was purchased from Molter (Heidelberg), blood-group O erythrocytes of P_1 and P_2 phenotype were kindly supplied by Dr G Wider (Austrian Red Cross). Stromata were prepared as in [8].

Haemagglutination and haemagglutination inhibition tests were performed as in [9], the P_1 cells being pretreated throughout with ficin. Protease activity in pronase-containing samples was destroyed prior to their use in haemagglutination-inhibition assays by heating them in a boiling water bath for 20 min. In the antibody absorption tests, 50 μl stromata and 20 μl antiserum were preincubated for 1 h at room temperature. The stromata were sedimented by centrifugation (20000 rev./min, 10 min), and the supernatant was tested for anti-P_1 activity by serum titration.

For pronase digestion, stromata from 15 ml packed erythrocytes were stirred with 2 mg pronase (Calbiochem, La Jolla CA) dissolved in 1 ml

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0.1 M Tris–HCl buffer (pH 7.5) containing 0.01 M $CaCl_2$ and 0.01% NaN_3 (37°C/2 h). The membranes were collected by centrifugation (10 min, 40000 rev./min) and partitioned twice between 1 ml water and 2 ml butanol-1. The suspension of the residual membranes in the water phase was diluted with 4 vol. distilled water and centrifuged as before. After one wash with distilled water the pellet was again treated with pronase (16 h) under the same conditions as above. Samples were drawn from the stromata after each step; the supernatants were concentrated to 100 μl in vacuo.

Fresh membranes obtained from 6 ml packed red blood cells were subjected to SDS–polyacrylamide gel electrophoresis according to [10] on 200 × 100 × 3 mm slab gels. The gels were stained with Coomassie blue (1.25 g in 455 ml methanol, 95 ml acetic acid and 455 ml water) and destained with several changes of 10% isopropanol/7% acetic acid; this procedure removed the majority of the detergent. Subsequently the bands were cut out and treated with 0.5 mg pronase/ml in the above Tris–HCl buffer overnight (37°C). The solution was filtered through filter paper to remove small gel particles, heated to destroy pronase, lyophilised, dissolved in 0.15 ml isotonic saline assayed for P_1 active material by haemagglutination-inhibition tests; bovine serum albumin had to be added to the test solution in order to bind traces of sodium dodecylsulfate [11] and thus prevent lysis of the erythrocytes.

Table 1

Changes in the blood-group P_1 activity of the ghosts from O,P_1- and O,P_2-erythrocytes after treatment with pronase and organic solvent

Pretreatment of the stromata used for absorption	Anti-P_1 serum titers after absorption with pretreated	
	P_1 stromata	P_2 stromata
Untreated	2	64
Pronase treated	2	32–64
Pronase and butanol treated	4–8	32–64
Pronase, butanol and again pronase treated	64	64

3. RESULTS AND DISCUSSION

Freshly prepared stromata pooled from blood samples of 5 blood-group P_1 donors were treated with pronase followed by partition between butanol-1 and water and a second digestion with pronase. After each step the membranes were tested for blood-group P_1 activity by their ability to absorb anti-P_1 antibodies. The first treatment of the stromata with pronase as well as extraction with organic solvent did not alter the P_1-activity of the membranes substantially (table 1). The second digestion with pronase, however, resulted in an almost complete loss of their capacity to absorb anti-P_1 antibodies.

When the supernatants were tested for P_1 activity, the results were in accordance with those obtained with the membranes: Only the material released by the second pronase treatment was able to inhibit the agglutination significantly (table 2). When stromata pooled from several P_2-erythrocytes were treated under the same conditions, no significant absorption of anti-P_1 could be observed at either level of degradation (table 1). These results show clearly that the majority of the P_1-determinant structures are localized on glycoproteins in the red cell membrane.

To gain more detailed information on the nature of the molecule(s) carrying the blood-group P_1 determinant, the membrane proteins were fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis. The bands were stained with Coomassie blue, cut out and digested with pronase. When the supernatants thereby obtained were tested in a haemagglutination inhibition

Table 2

Blood-group P_1 activity of the material liberated from the P_1 stromata by treatment with pronase and organic solvent as tested by the inhibition of the agglutination of P_1 erythrocytes by anti-P_1 serum

Sample		Inhibition titer
1st pronase supernatant		2
Extraction	butanol phase	0
	water phase	0
2nd pronase supernatant		32

Table 3

Blood-group P_1 activity of membrane components separated by SDS–polyacrylamide gel electrophoresis as tested by the inhibition of the agglutination of P_1 erythrocytes by anti-P_1 serum

Membrane component	Inhibition titer
Bands 1 and 2	2
Band 3	2
Bands 4.1 and 4.2	4
Band 4.5	32
Band 5	2
Band 6	2
Gel front (dye)	0

assay, the P_1 activity was localized sharply in the region of band 4.5 (table 3). According to [12], however, band 3 is frequently cleaved during the preparation procedure for electrophoresis by a stromal protease yielding fragments that comigrate with band 4.5. Therefore it cannot be completely excluded that the P_1-active material in its native state might in reality have been a band 3 glycoprotein rather than a band 4.5 substance.

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