Glycoproteins with Galα4Gal Are Absent from Human Erythrocyte Membranes, Indicating That Glycolipids Are the Sole Carriers of Blood Group P Activities*

(Received for publication, November 1, 1993, and in revised form, February 10, 1994)

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The antigenic determinants of the blood group P family (P, P*, Pk, LKE), and one antigen of unassigned high incidence have been analyzed on replicas of sodium dodecyl sulfate-polyacrylamide electrophoresis gels using specific reagents (Escherichia coli HB101/pDC1 expressing the Pap gene and monoclonal antibodies with specificities for P, and Pk antigens). No binding to glycoproteins was detected with any of these ligands when the ghosts had been pretreated with butanol to remove glycolipids. Therefore, all antigenic determinants of the P blood group family on human red cells are exclusively expressed in glycolipids and are absent from glycoproteins.

In the present study, specific monoclonal antibodies and a recombinant Escherichia coli strain expressing an adhesin that is specific for Galα4Gal-containing sequences were used to analyze glycoproteins of human erythrocyte membranes (ghosts) on replicas of SDS-PAGE gels (blots). No activity was detected, indicating that glycolipids are the sole carriers of Galα4Gal-based blood group determinants on human red cells.

MATERIALS AND METHODS

Neoglycoproteins—Galα4Galβ-PAP-HSA, Galβ3Galβ4Glcβ-CETE-BSA, Galα4Galβ4GlcNAcβ-CETE-BSA, GalNAcβ3Galα4Galβ4Glcβ-APD-HSA, and Manβ3Manβ4GlcNAcβ-APD-HSA were obtained from Bio-Carb (Lund, Sweden). Chemical spacers used for the synthesis of neoglycoproteins (Bio-Carb) were: PAP, p-aminophenyl; CETE, 2-(2-carboxyethoxyethyl)isothiouronium; and APD, acetylated phenylenediamine. A bracket means reduced (open ring) sugar. 0.5 μg of each was used for SDS-PAGE.

Samples of Glycolipids—P glycolipid (Galα4Galβ4GlcNAcβ3Galβ4Glcβ-Cer) and Pk glycolipid (Galα4Galβ4Glcβ-Cer) were prepared and structurally characterized by mass spectrometry, nuclear magnetic resonance spectroscopy, and degradation studies as described elsewhere (8). Dog intestine total neutral glycolipids were prepared as described previously (9). Forsmian glycolipid (GalNAcβ3Galα4Galβ4Glcβ-Cer) was the main glycolipid of dog intestine (10). The isolated and purified glycolipids were dissolved in 50 mm Tris-HCl buffer (pH 8.0) containing 2.5% SDS and 5% 2-mercaptoethanol.

Bacteria and Labeling—The recombinant E. coli HB101/pDC1 expressing the Pap gene cloned from the uropathogenic E. coli IA2 (11) was used, having specificity for both terminal and internal Galα4Gal sequences (12, 13). The bacteria were cultivated overnight at 37 °C on colonization factor antigen agar plates, to which 20 μl of [3H]methionine, 15 μCi/ml (Amersham, Buckinghamshire, U. K.), was added to label the bacteria. After growth, the bacteria were harvested by centrifugation, washed three times in phosphate-buffered saline (pH 7.4), and finally resuspended in the same buffer to give suitable radioactivity counts. About 10^6 cpm was incorporated into 10^9 cells/ml, giving 100 bacteria/cpm.

Monoclonal Antibodies and Iodination—Three monoclonal antibodies with specificities against P, antigen (PO01), Pk antigen (PO002) and Galα4Galβ-Cer, galabiosyllceramide (MC2102, 87.5/C11), respectively, were obtained from Bio-Carb. The specificities of these antibodies have been checked by binding to isolated glycolipids separated on thin-layer plates. Rabbit anti-mouse immunoglobulin antibody was purchased from DAKO (Copenhagen, Denmark). Iodination was done with Na[125]I, 100 μCi/ml (Amersham), according to the IODO-GEN protocol of the manufacturer (Pierce Chemical Co.). The free 125I was removed by gel filtration chromatography using a Sephadex G-50 PD-10 column (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). The buffer for the column equilibration and the protein separation consisted of 20 mm Hepes/ROH, 100 μm NaCl, 0.01% Tween 20 (pH 7.4).


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* The work was supported by Swedish Medical Research Council Grant 3967 and by Symbicom Ltd., Umeå, Sweden. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Absence of Blood Group P Activities in Glycoproteins

Preparation of Human Erythrocyte Membranes—Fresh human erythrocytes clinically identified as blood group P(+) or P(-) were obtained from the Blood Bank, Sahlgren's Hospital, Göteborg. Membranes (unsealed ghosts) were prepared according to Steck and Kant (14). Red cells were washed three times in 50 mM sodium phosphate buffer with 150 mM NaCl (pH 7.0) and then lysed by rapid and thorough mixing in 1 volume of packed cells with approximately 40 volumes of 50 mM sodium phosphate buffer (pH 8.0). The membrane ghosts were pelleted by centrifugation at 22,000 xg for 20 min before electrophoresis. A gradient gel of 8-25% was used, and the samples were heated at 95 °C for 10 min, and centrifuged at 12,000 xg for at least 1.5 h. Then the membrane was incubated with either E. coli HB101/pDCl or the monoclonal antibodies for overlay. The monoclonal antibodies were diluted in overlay solution (50 mM Tris-HCl containing 1% BSA, 200 mM NaCl, and 0.05% Tween 20 (pH 8.0)) to a final concentration of 1-2 μg/ml. After 2 h, the membrane was washed by washing solution (50 mM Tris-HCl containing 200 mM NaCl, 0.05% Tween 20 (pH 8.0)). The second labeled anti-mouse antibody, with a final concentration of 1-2 μg/ml and radioactivity counts of 3,000-4,000 cpm/ml, was added for 2 h followed by repeated washing. Then the membrane was dried at room temperature for at least 0.5 h and exposed to x-ray film (Kodak) overnight.

RESULTS

The results from the binding of E. coli and the three monoclonal antibodies to the synthetic neoglycoproteins are summarized in Table I. As expected, because of its ability to recognize both a terminal and an internal Galα4Gal sequence (12, 13), the bacteria bound to all of the four Galα4Gal-containing neoglycoproteins but not to the Manα4Glαβ4GlcNAc-APD-HSA used as a negative control. However, the monoclonal antibodies which all bind to terminal sequences, which is the rule (16), bind with a different pattern. Thus, the neoglycoprotein GalNαβ3Galβ4Galβ4Glαβ4Glc-APD-HSA was not bound by any of the three different antibodies used in this study. The antibody against the P, antigen did not bind to the simple neoglycoprotein Galα4Galβ-P-P-HSA.

The Pα and Pβ glycolipids and dog intestine total neutral glycolipids added to the nitrocellulose membrane were all bound by E. coli. However, only Pβ glycolipid can react with all three monoclonal antibodies. The Pα glycolipid reacted with anti-Pα, and anti-Pβ antibodies but not with anti-Galα4GalβCer antibody. The dog intestine glycolipids was not bound by any of these antibodies.

The binding of E. coli to blots of human erythrocyte ghosts was observed not only for blood group P(+) but also for the P(-). However, the binding was in the gel front, which was not stained by Coomassie Blue R-350, and the positive binding disappeared if the erythrocyte ghosts were pretreated thoroughly by butanol (see Fig. 1). Except for this, no binding of E. coli to glycoproteins of human ghosts was observed.

The antibodies did not bind on blots of ghost glycoproteins, either from blood group P(+) or P(-), regardless of butanol extraction (see Fig. 2).

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<th>Table I Binding of E. coli HB101/pDCl and three monoclonal antibodies to human erythrocyte membranes and some references on blots of nitrocellulose membrane</th>
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absence of blood group P activities in glycoproteins residues after glycolipid extraction of pooled samples of various blood groups (data not shown), as well as on the human kidney and granulocyte residues (see Fig. 2).

The ceramide glycanase treatment of ghosts reduced the strength of, but could not eliminate, the binding of the E. coli on SDS-PAGE. However, the positive components of the blood group P(+) and P(-) erythrocyte membranes were all chloroform: methanol-soluble. The chloroform: methanol-insoluble fraction did not contain any binding component. Furthermore, the positive binding was completely abolished after the chloroform: methanol-soluble fraction was treated by ceramide glycanase (see Fig. 3). The fact that the binding spot was a little bit higher in lane 1 than in lane 3 was caused by a torsion in the margin of the gel when blotted to the nitrocellulose membrane.

**DISCUSSION**

The human blood group P system and P, Pk, and LKE collection antigens are chemically based on the Galα4Galβ sequence. The P, antigen (Galα4Galβ4GlcNAcβ3Galβ4GlcβCer) is present on red cells of the P, and Pk phenotype, the P antigen (GalNAcβ3Galα4Galβ4GlcβCer, globotetraosylceramide or globoside) is present on P, and P, cells, and the Pk antigen (Galα4Galβ4GlcβCer, globotriaosylceramide) is present in low amounts on P, and P, cells but enriched in cells of the infrequent phenotypes P1k and P2k. The LKE antigen is present in almost all red cells except those of the rare phenotypes p or P*, and in about 2% of P positive samples (17). The murine monoclonal antibody MC813-70, which defines the stage-specific embryonic antigen 4, was found to react with LKE(+) but not LKE(-) red cells (18). Therefore, the LKE antigenic structure should be the same as that of stage-specific embryonic antigen 4, NeuAcα3Galβ3GalNacβ3Galα4Galβ4GlcβCer (17, 18). The very rare phenotype p (small p) is devoid of all of these antigens but enriched in Galβ4GlcβCer (lactosylceramide).

Because of the ability of bacteria to recognize both terminal and internal sequences (19, 20), it is not surprising that the recombinant E. coli bound to both P(+) and P(-) erythrocyte samples, since the globoside (P antigen) is the major glycolipid in human erythrocytes and present on all common red cells (21). The positive binding of E. coli to P(+) and P(-) samples resulted from the presence of Galα4Gal-containing glycolipids, since the binding disappeared if the samples were thoroughly treated by butanol to remove glycolipids before electrophoresis.
Previously, it was claimed that erythrocyte membrane glycoproteins were carriers of blood group P, determinants (5), which means that Galα4Gal-containing glycoproteins exist in human P (+) erythrocytes. It was said that the Pronase treatment of butanol-extracted membranes liberated P, determinants from the ghosts, and the P, activity found in the band 4.5 region on SDS-PAGE was assayed by cutting out the gel band and recovering the materials, which were then subjected to a hemagglutination inhibition test (5). However, we could not find any binding of these Gala4Gal-specific reagents to band 4.5, which was reported to contain P, activity. One notable result from the present study was that the glycolipids may still remain in the residual membranes (see Fig. 1) if the ghosts are treated only by butanol (5), but they are completely removed first when the ghosts are dissolved by SDS followed by treatment with butanol.

The clinically defined P, (+) erythrocyte samples on the replicas of SDS-PAGE gels were only bound by E. coli but did not react with the Gala4Gal-specific antibodies, even the anti-P, antibody. The reason may be that the epitopes of glycolipids are difficult to detect in mixed micelles, since in the electrophoresis gel, all glycolipids migrate together in the front of the gel. To compare the electrophoretic behavior and binding property of glycolipids on SDS-PAGE, isolated glycolipids, including P, and P, glycolipids, and dog intestine total neutral glycolipids, were subjected to electrophoresis, blot, and overlay as for glycoproteins. All of these glycolipids were bound by E. coli. However, only P, glycolipid reacted with all three monoclonal antibodies. The P, glycolipid reacted with anti-P, and anti-P, antibodies but not with anti-Galα4GalβCer antibody. Notably, the dog intestine total neutral glycolipids, of which globotriaosylceramide and galabiaosylceramide gave strong binding on thin-layer plates (5), did not bind to any of these antibodies on the nitrocellulose membrane. This result may indicate that some epitopes of glycolipids might be masked by one another because of the mixed micelles of component glycolipids in the electrophoresis situation.

To clarify further the chemical characteristics of the positive binding components of human erythrocyte membrane by Gala4Gal-specific reagents, we treated the erythrocyte membrane and its chloroform:methanol-soluble fraction with ceramide glycanase. The chloroform:methanol-insoluble fraction did not contain any positive binding component at all. Instead, all positive binding components of blood group P, (+) and P, (-) erythrocyte membranes were chloroform:methanol-soluble. Moreover, the binding of the E. coli to the chloroform:methanol-soluble fraction was completely abolished after the fraction was treated by ceramide glycanase, as shown in Fig. 3. This clearly demonstrated that the positive binding was due to glycolipids rather than glycoproteins. The ceramide glycanase hydrolyzes the linkage between the ceramide and the sugar chain in various glycosphingolipids and releases the intact oligosaccharides (22), which were then washed away during the subsequent blot and overlay procedures. In the case of the intact erythrocyte membrane (ghost) treated by the ceramide glycanase, the poor solubility of the ghost in the lower concentration of sodium cholate led to a poor accessibility of the enzyme to the substrates. Thus the positive binding was only reduced but not completely abolished (see Fig. 3).

Since approximately 75% of white individuals possess the P, antigen (21), the mixed red cell membrane residues pooled from many hundred individuals with the same blood group O, A, B, or AB must include P, (+) individuals. However, as no binding to glycoproteins of the four groups of red cell membrane residues was observed, it is unlikely that Gala4Gal-containing glycoproteins exist on human red cells.

Using the recombinant E. coli strain as a specific binding reagent, we have found Gala4Gal-containing glycoproteins expressed in some animal tissues (2) and human tumor tissues (3), to which the monoclonal antibodies recognizing Gala4Gal-containing carbohydrate sequences could not bind. The recombinant E. coli strain expressing the specific adhesin that binds Gala4Gal is more useful than the monoclonal antibodies for screening Gala4Gal-containing glycoproteins, since it is able to recognize both a terminal and an internal disaccharide.

Acknowledgments—We are indebted to Maan Abul Milh for cultivation and labeling E. coli, to Britt-Inger Marklund for the recombinant E. coli, and to Bo E. Samuelsson for P, (+) and P, (-) blood samples. We are grateful to Boel Lanne and Halina Miller-Podraza for helpful discussions during the work.

REFERENCES


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