Identification and immunochemical characterization of the human erythrocyte membrane glycoproteins that carry the Xg^a antigen

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When the membrane components of Xg(a+) erythrocytes were separated by electrophoresis and immunoblotted with an anti-Xg^a of human origin, two diffuse bands of approximate M_r 22 000–25 000 and 26 500–29 000 were stained. These reactive components were not evident in membranes from proteinase-treated Xg(a+) erythrocytes. Neuraminidase treatment of erythrocytes before immunoblotting resulted in one diffuse Xg^a-reactive band, the leading edge of which had a slightly increased mobility corresponding to a decrease in M_r of approx. 1500. These results suggest that the membrane components that carry Xg^a are sialoglycoproteins. A genetic relationship exists between Xg^a and the antigen recognized by the monoclonal antibody 12E7. An immunochemical comparison of the structures that carry the Xg^a and 12E7 antigens demonstrated that they differ in M_r and in the way in which they are modified by neuraminidase. This is in accord with evidence that Xg^a and 12E7 are the products of two separate structural loci.

INTRODUCTION

The human erythrocyte antigen Xg^a is the product of an X-chromosome-borne locus (Mann *et al.*, 1962). No antigen antithetical to Xg^a has been found, so that the phenotypes are Xg(a+) and Xg(a−). About 89% of females and 66% of males are Xg(a+) (Race & Sanger, 1975). Xg^a is the dominant gene that encodes the Xg^a antigen, and a silent allele Xg is postulated to explain the absence of Xg^a from Xg(a−) individuals. The presence of the antigen on erythrocytes is established by haemagglutination tests with human sera that contain anti-Xg^a alloantibodies.

A mouse monoclonal antibody, 12E7, raised against lymphocytes from a patient with acute T-cell lymphocytic leukaemia (Levy *et al.*, 1979), defines an antigen that is the product of the *MIC2* structural gene (Goodfellow *et al.*, 1980, 1983). This is the only expressed human gene known to be carried by both X and Y chromosomes (Goodfellow *et al.*, 1985). Expression of the *MIC2* gene results in the presence of 12E7 antigen on many human tissues (Goodfellow, 1983), but it is only on erythrocytes that 12E7 antigen expression is variable, the level of expression being related to the Xg blood groups (Goodfellow & Tippett, 1981). All Xg(a+) individuals are high expressors of 12E7 antigen, and all Xg(a−) women are low expressors. However, the antibody 12E7 is not anti-Xg^a because Xg(a−) men may be high or low expressors of 12E7. To explain this relationship, the existence of three closely linked loci, *XGS*, *XGR* and *MIC2*, has been proposed (Goodfellow *et al.*, 1987). *XGS* and *MIC2* are the structural loci encoding Xg^a and 12E7 antigens respectively. *XGR* is a regulatory locus occurring on both X and Y chromosomes. It is polymorphic, with two alleles, A and B, that control in *cis* expression of *XGS* and *MIC2*. In *cis*, the A allele induces Xg^a antigen and high-level 12E7 antigen expression from the *XGS* and *MIC2* loci respectively. The B allele, in *cis*, results in failure to produce Xg^a antigen and low-level 12E7 antigen expression.

Immunochemical analysis of the membrane structure that expresses the 12E7 antigen has shown it to be a sialoglycoprotein of approximate M_r 32 500 (Banting *et al.*, 1985). In the present paper the membrane components that carry the Xg^a antigen are identified, and the characteristics of the Xg^a and 12E7 components are compared.

MATERIALS AND METHODS

Xg^a-typed erythrocytes, S−s−U− erythrocytes (donor AB) and Tn erythrocytes (donor JA) were available through the United Kingdom rare cell exchange scheme. Tn erythrocytes (donor Ba) were provided by Dr. C. Hodson and Dr. D. Lee, Lancaster Regional Transfusion Centre, Lancaster, U.K. The serum containing anti-Xg^a was from a multi-transfused renal-dialysis patient (CG) awaiting transplant and was available at the Wessex Regional Blood Transfusion Centre. Anti-Xg^a antibodies were purified for immunoblotting by acid elution from digitonin-lysed erythrocytes (Jenkins & Moore, 1977). An acid eluate containing anti-s (donor SAB), which immunoblots two bands of M_r 25 000 (leading edge) and 50 000, has been described previously (Herron *et al.*, 1989). R 1.3, a mouse monoclonal antibody that reacts with the N-terminal portions of both sialoglycoproteins α and δ (Anstee & Edwards, 1982), was provided by Dr. D. Anstee, South Western Regional Blood Transfusion Centre, Bristol, U.K. The mouse monoclonal antibody 12E7 was kindly given by Dr. P. N. Goodfellow, Imperial Cancer Research Fund, London, U.K.

Neuraminidase (type V; from *Clostridium perfringens*) and tosylphenylalanylchloromethane- ('TPCK')-treated trypsin were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Papain, Pronase and crystalline α -chymotrypsin were from BDH Chemicals, Poole, Dorset, U.K. The methods used for proteinase treatment of erythrocytes have been previously described (Herron *et al.*, 1989). Neuraminidase was diluted to 1 unit/ml in

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0.15 M-NaCl, pH 6.0, containing 1 mM-CaCl₂. Erythrocytes were washed three times in a large excess of 0.15 M-NaCl, with centrifuging at 2000 *g* for 3 min after each wash. Packed washed erythrocytes (1 vol.) were resuspended in 0.15 M-NaCl containing 1 mM-CaCl₂ (3 vol.) and incubated with the diluted neuraminidase (4 vol.) for 30 min at 37 °C.

The methods for preparation of erythrocyte ghosts and cytoskeletons and details of the immunoblotting procedure have also been described previously (Herron *et al.*, 1989). Immunoblotting was modified by the use of a 3% (w/v) stacking gel, and 3% (v/v) bovine serum albumin in 0.3% (v/v) Tween/0.135 M-NaCl/0.015 M-sodium phosphate buffer, pH 7.3, as the blocking agent and as diluent for antiglobulin reagents.

RESULTS AND DISCUSSION

Antibodies with anti-Xg^a specificity were identified in the serum of patient CG by haemagglutination. In the antiglobulin test, positive reactions were obtained with the erythrocytes from 19 different Xg(a+) donors and negative reactions with those from 14 Xg(a-) donors. The antibody was IgG and reacted to a titre of 8 with untreated erythrocytes but failed to react with erythrocytes pretreated with trypsin, α -chymotrypsin, papain or Pronase.

Electrophoresis of the SDS-solubilized membranes from Xg(a+) erythrocytes, followed by immunoblotting with anti-Xg^a (CG) under reducing or non-reducing conditions, demonstrated two diffuse bands of approximate *M_r* 22 000–25 000 and 26 500–29 000 (four determinations). A component of approximate *M_r* 23 500–25 000 (four determinations), at the trailing edge of the lower-*M_r* band, was strongly stained (Fig. 1, lanes, a, f and k).

Immunostaining occurred with membranes prepared from nine different Xg(a+) donors but not with the membranes from six Xg(a-) donors (*P* = 0.0002 by using Fisher's 'exact' method for 2 × 2 tables).

The separated membrane components from Xg(a+) erythrocytes that had been pretreated with trypsin, α -chymotrypsin, papain or Pronase failed to bind anti-Xg^a (CG) (results not shown). This is consistent with the results of haemagglutination tests in this and other reports (Habibi *et al.*, 1979; Nakajima *et al.*, 1979) that have demonstrated the proteinase-sensitivity of the Xg^a antigen. When intact erythrocytes were treated with neuraminidase and the separated membrane components immunoblotted with anti-Xg^a (CG), a diffuse band of *M_r* 20 750–23 250 (mean of two determinations) was stained (Fig. 1, lane g). The leading edge of this band showed a slight increase in mobility corresponding to a decrease in *M_r* of approx. 1500 when compared with the leading edge of the low-*M_r* band obtained with untreated erythrocytes. These results suggest that the erythrocyte membrane components that carry Xg^a are sialoglycoproteins. A normal pattern of reactivity was observed when Tn erythrocytes (JA and Ba) were immunoblotted with anti-Xg^a (CG) (results not shown). Tn erythrocytes are abnormal in that they are deficient in the β -3-galactosyltransferase necessary for the complete synthesis of the *O*-glycosidically linked sialotetrasaccharides present on erythrocyte sialoglycoproteins (Cartron *et al.*, 1978). These abnormal proteins will have an altered electrophoretic mobility, and this has led to the use of Tn erythrocytes in assessing the *O*-glycan content of membrane glycoproteins (Spring *et al.*, 1987, 1988). However, interpretation of the results obtained with Tn erythrocytes may be complicated by the observation that the removal of the galactose-bound sialic acids from *O*-

Fig. 1. Immunostaining of human erythrocyte membrane glycoproteins by anti-Xg^a (CG) and 12E7 monoclonal antibody after electrophoresis and transfer to nitrocellulose sheets

Erythrocyte membrane and cytoskeleton samples were solubilized under reducing conditions and immunoblotted. Nitrocellulose strips from different experiments are arranged in groups. Lanes a-c, f, g, k and l, immunoblots with anti-Xg^a (CG): lanes a, f and k, Xg(a+) membranes; lane b, Xg(a-) membranes; lane c, S-s-U-, Xg(a+) membranes (donor AB); lane g, membranes from neuraminidase-treated Xg(a+) erythrocytes; lane l, cytoskeletons from Xg(a+) erythrocytes. Lanes h, i, m and n, immunoblots with 12E7: lanes h and m, Xg(a+) membranes; lane i, membranes from neuraminidase-treated Xg(a+) erythrocytes; lane n, cytoskeletons from Xg(a+) erythrocytes. Lanes d and e, immunoblots with R 1.3: lane d, S-s-U- membranes (donor AB); lane e, Ss membranes. Lane j, immunoblot of ss membranes with anti-s (SAB). Abbreviation: SGP δ , sialoglycoprotein δ .

glycosidically linked tetrasaccharides causes a decrease in electrophoretic mobility whereas the absence of both galactose-linked and *N*-acetylgalactosamine-linked sialic acids results in an increased mobility (Gahmberg & Andersson, 1982). Furthermore, a normal pattern of reactivity with Tn erythrocytes does not appear to exclude the presence of *O*-glycans (Spring *et al.*, 1988).

Because sialoglycoprotein δ migrates to the same region as the glycoproteins carrying Xg^a, investigations were performed to exclude any relationship between these components. The mobilities of sialoglycoprotein δ and the Xg^a proteins were compared by immunoblotting adjacent lanes with anti-s (SAB) and anti-Xg^a (CG). From this comparison, it was apparent that the sialoglycoprotein δ band, detected by anti-s (SAB), coincided with the region between the two diffuse bands immunoblotted by anti-Xg^a (CG). This was a consistent observation in four separate experiments, one of which is illustrated (Fig. 1, lanes j and k). Erythrocytes with the rare blood-group phenotype S-s-U- lack sialoglycoprotein δ (Dahr *et al.*, 1975; Anstee *et al.*, 1979). Membranes from S-s-U- erythrocytes (AB) were immunoblotted with anti-Xg^a (CG) and the monoclonal antibody R 1.3 (Fig. 1, lanes c and d). The absence of sialoglycoprotein δ from the membranes was confirmed by the negative reaction with R 1.3. The positive immunoblot with anti-Xg^a (CG) therefore indicated that the Xg^a proteins are not derived from sialoglycoprotein δ . It is also apparent from this blot that, in the absence of sialoglycoprotein δ , anti-Xg^a (CG) binds to one continuous band of M_r 22 500–28 000, which suggests that, in immunoblots with normal membranes, sialoglycoprotein δ in some way partially inhibits or blocks the reaction of anti-Xg^a (CG) with the separated membrane components.

The Xg^a glycoproteins do not bind strongly to the cytoskeleton since the majority of the reactive components are easily extracted from the erythrocyte membranes with low concentrations (0.1%, v/v) of Triton X-100 (Fig. 1, lane l). In this respect the Xg^a glycoproteins differ from sialoglycoproteins β and γ (Owens *et al.*, 1980; Anstee *et al.*, 1984).

The genetic relationship that exists between Xg^a and 12E7 suggested an immunochemical comparison of the structures carrying these antigens. The separated membrane components of Xg(a+) erythrocytes were immunoblotted with 12E7. The leading edge of the 12E7 band (Fig. 1, lanes h and m) had an M_r of 33 250 (mean of two determinations). This is in close agreement with the previously determined M_r of 32 500 for this protein (Banting *et al.*, 1985). When neuraminidase-treated erythrocytes were immunoblotted with 12E7, two bands were stained (Fig. 1, lane i), the increased mobility of the lower- M_r band representing a decrease in M_r of approx. 6000 following neuraminidase treatment. This pattern of reactions resembles the pattern reported in previous investigations (Latron *et al.*, 1987). Similarly, the absence of the 12E7 glycoprotein from cytoskeletons prepared by extraction of erythrocytes with 0.1% (v/v) Triton X-100

(Fig. 1, lane n) confirms previous results (Latron *et al.*, 1987).

The observations reported in this paper show that the structures carrying 12E7 and Xg^a differ in M_r and in the way in which they are modified by neuraminidase. This is consistent with evidence that Xg^a and 12E7 have separate structural loci (Goodfellow *et al.*, 1987).

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