Immunochemical characterization of the human blood cell membrane glycoprotein recognized by the monoclonal antibody 12E7

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The 12E7 murine monoclonal antibody recognizes a protease-sensitive component of human red cells, platelets and lymphocytes which could not be detected on granulocytes. Scatchard analyses indicated that the ^{125}I -labelled antibody binds to 1000, 4000 and 27000 antigen sites on each red cell, platelet and lymphocyte respectively, with a binding constant ranging from 4×10^7 to $9 \times 10^7 \text{ M}^{-1}$. The membrane components recognized by the monoclonal antibody were characterized by immunostaining on nitrocellulose sheets. A 28 kDa sialoglycoprotein was visualized following electrophoretic transfer of the red cell and lymphocyte membrane proteins separated by SDS/polyacrylamide-gel electrophoresis. Another component of 25 kDa was also clearly identified in the lymphocyte and platelet lysates, but was barely detectable in the red cell membrane preparations. Enzyme treatment of intact platelets, as well as analysis of the membrane and cytosolic preparations from these cells, have shown that the 25 kDa component was of cytoplasmic origin. The mobility of the 28 kDa membrane component is decreased following neuraminidase treatment of intact blood cells, but these cells still react normally with the monoclonal antibody, indicating that sialic acids are not required for binding. The 28 kDa component is present on red cell membranes prepared from S-s-U-, En(a-) and Gerbich(-) individuals, demonstrating that it is a new sialoglycoprotein not derived from glycoporphins A, B, C or D. The 28 kDa component was totally solubilized with 0.1 % Triton X-100 from red cell membranes and behaves like the other red cell membrane sialoglycoproteins since it was extracted in the aqueous phase following chloroform/methanol/water or butanol/water partitionings. The 28 kDa component could be partially purified by h.p.l.c. gel permeation chromatography and preparative SDS/polyacrylamide-gel electrophoresis. The material finally obtained strongly inhibits the 12E7 monoclonal as well as human anti-Xg^a antibodies, suggesting either that the 28 kDa glycoprotein carries both antigens or that the 12E7 and Xg^a-active molecules copurified.

INTRODUCTION

The 12E7 antigen is defined by a murine monoclonal antibody raised against lymphocytes from a patient suffering from a T cell acute lymphocytic leukaemia (Levy *et al.*, 1979). The antigen is not only present on thymocytes but has a large tissue distribution, particularly in human, chimpanzee and gorilla species. It is however absent from a number of animal species (Goodfellow, 1983). The study of human-rodent hybrids has demonstrated that expression of the 12E7 antigen is under the control of a gene called *MIC2*, and located on the short arm of the X chromosome (Xp22.32-Xpter) and that an additional controlling locus is also present on chromosome Y (euchromatin region Yq11-Ypter) (Goodfellow *et al.*, 1983, 1984; Buckle *et al.*, 1985). Such findings suggest that the genes regulating 12E7 expression are located within the region of sex chromosomes paired during meiosis (Pearson & Bobrow, 1970; Solari, 1974) and that the X and Y chromosomes might share at least one common functional gene. Biochemical analyses have indicated that the 12E7 antigen of lymphocytes and human cell lines is associated with a 29–32.5 kDa polypeptide (Levy *et al.*, 1979; Banting *et al.*, 1985) but this material was not purified and characterized.

The *MIC2* gene is close to the Xg locus, and

investigations by Goodfellow & Tippet (1981) have shown sex limitation of 12E7 expression on human red cells, related to a complex quantitative polymorphism involving the blood-group-Xg phenotypes, which is presumably regulated by a postulated Y-linked locus called Yg. Accordingly, all Xg(a+) individuals are high level expressors of 12E7 whereas all Xg(a-) females are low expressors. Xg(a-) males, however, belong to both high and low expressors of 12E7.

Since the Xg locus is acting as a controlling locus for the 12E7 antigen polymorphism it was of interest to study the relationship between Xg^a and 12E7 antigens. For this purpose, we took advantage of the 12E7 monoclonal antibody to identify and partially purify the glycoproteins which carry the 12E7 antigen.

MATERIALS AND METHODS

Reagent grade chemicals were used throughout. Pronase from *Streptomyces griseus* (8 DMC units/mg) was obtained from Serva. Trypsin [1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK')-treated] (182 units/mg) and α -chymotrypsin (53 units/mg) were purchased from Worthington. Neuraminidase from *Vibrio cholerae* (1 unit/ml) was obtained from Behringwerke AG

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(Marburg-Lahn, Germany). The chemicals used for polyacrylamide-gel electrophoresis in SDS have been described previously (Blanchard *et al.*, 1982). Na¹²⁵I (15 mCi/ μ g of iodine) and ¹²⁵I-labelled goat anti-(mouse IgG)(10 μ Ci/ μ g) were bought from Amersham International (U.K.). ¹²⁵I-labelled protein A (6 μ Ci/ μ g) was from the Centre National de Transfusion Sanguine (Orsay, France) and NaB³H₄ (16 μ Ci/nmol) was purchased from the Centre d'Energie Atomique (Saclay, France).

Red cells from adult donors typed for their high and low expression of 12E7 antigen using a radioimmunoassay described under Table 1 (Casanova-Bettane *et al.*, 1981) were from the Centre National de Transfusion Sanguine (Paris, France). Red cells from individuals of rare blood group phenotypes: En(a-) (M.E.P. and G.W.), S-s-U- (Fav.) and Ge(-) (Ped.) and the human anti-Xg^a serum were from the Centre National de Référence sur les Groupes Sanguins (Paris, France). The fetal red cells were obtained from Dr. F. Forestier (Hôpital de Bon Secours, Paris, France).

Red cells were labelled with NaB³H₄ after periodate oxidation according to Gahmberg *et al.* (1976). Treatments of erythrocytes with Pronase, papain, trypsin and chymotrypsin were performed according to Anstee & Edwards (1982) and Blanchard *et al.* (1982). Desialylated red cells were obtained after 30 min incubation at 37 °C with 0.025–0.5 units of neuraminidase from *Vibrio cholerae* in 50 mM-acetate buffer, pH 5.5, containing 9 mM-CaCl₂ and 150 mM-NaCl. The supernatant obtained after treatment of 30 ml of packed red cells with 4 μ mol of trypsin was treated with a 10-fold molar excess of tosyl-lysylchloromethane ('TLCK'), filtered on Millipore membrane (0.22 μ m), dialysed against distilled water for 24 h at 4 °C and lyophilized.

Membranes from washed packed red cells were prepared by hypo-osmotic lysis according to Steck & Kant (1974) and solubilized in 30 mM-Tris/HCl (pH 6.8)/3 mM-EDTA 15% (w/v) SDS (buffer A) to obtain a 5% final SDS concentration.

Platelets and granulocytes were prepared from blood (9 vol.) collected on 1 vol. of 5% (w/v) EDTA in 150 mM-NaCl (Nurden *et al.*, 1981; Cartron *et al.*, 1983). Platelets were treated with trypsin or chymotrypsin according to Phillips (1972) and both membrane and cytoplasmic fractions from platelets were prepared by the glycerol technique of Barber & Jamieson (1970). Lymphocytes free of contaminating platelets were prepared as described (Cartron *et al.*, 1980) from defibrinated blood collected on glass beads (Prolabo, Paris, France, 2 mm diameter). Lymphocytes and granulocytes were disrupted by 30 min incubation at 0 °C in 38 mM-Tris/HCl, pH 7.2, containing 10 mM-glycine, 1 mM-CaCl₂, 1 mM-phenylmethanesulphonyl fluoride, 5 mM-N-ethylmaleimide and 1% (w/v) Triton X-100 (Andersson & Gahmberg, 1978). The lysates were first centrifuged in the cold room at 30000 g for 15 min and boiled with one-third volume of buffer A. Platelets were prepared for gel electrophoresis as described by Nurden *et al.* (1981).

The SDS lysates obtained from red cells, platelets, lymphocytes and granulocytes were analysed by SDS/polyacrylamide-gel electrophoresis in the discontinuous buffer system of Laemmli (1970) as previously described (Blanchard *et al.*, 1982). Glycoproteins were stained with the periodate/Schiff technique (Fairbanks *et al.*, 1971). Membrane proteins from red cells, lymphocytes,

platelets and granulocytes were transferred to 0.10 μ m-pore nitrocellulose sheets (Schleicher und Schüll, Dassel, Germany) using the Transblot apparatus from BioRad according to a method derived from Towbin *et al.* (1979), as briefly described in the legend to Fig. 2. The nitrocellulose sheets were then saturated with 10 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl and 0.6% (w/v) gelatine (Prolabo, France) and incubated with a 100-fold dilution of the 12E7 monoclonal antibody (ascites fluid) in the same buffer. After washing off the excess of antibody, specific binding was estimated by staining with a peroxidase-labelled sheep anti-(mouse immunoglobulin)(Institut Pasteur, Paris) as previously described (Edelman *et al.*, 1984).

Purified IgG (500 μ g) from the murine monoclonal antibody 12E7 was radio-iodinated using Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril; Pierce) as oxidizer. The reaction was carried out in 2 ml plastic flasks coated with 100 μ g of Iodogen (Fraker & Speck, 1978), stored dry and washed with phosphate-buffered saline (10 mM-phosphate/150 mM-NaCl, pH 7.2) prior to use. The monoclonal antibody (500 μ l) and Na¹²⁵I (100 μ Ci) solutions were reacted in the presence of Iodogen for 10 min at room temperature. Free iodine was separated from labelled antibody (specific radioactivity 0.7 μ Ci/ μ g of protein) by gel filtration on a 10 ml Sephadex G-25 column equilibrated with 0.15 M-NaCl.

Binding studies of the ¹²⁵I-labelled monoclonal antibody to red cell, platelet, lymphocyte and granulocyte suspensions were carried out in plastic tubes containing 0.1 ml of 0.4% (w/v) bovine serum albumin in phosphate-buffered saline, 0.02–10 μ g of ¹²⁵I-labelled 12E7 monoclonal antibody and an appropriate number of washed red cells (5×10^7 platelets, 5×10^6 granulocytes or 5×10^5 lymphocytes). Following 1 h incubation at room temperature with continuous shaking, the cells were washed three times with phosphate-buffered saline/albumin to remove unbound antibody. The cell pellets were then transferred to a clean plastic tube and the amount of 12E7 antibody bound was determined by ¹²⁵I counting. All assays were conducted in duplicate. Estimates of the number of antibody molecules bound per cell, as well as equilibrium constants, were from Scatchard (1949) plot analysis.

Sialoglycoproteins were extracted from red cell membranes using the phenol/saline (Glöckner *et al.*, 1976), Lis/phenol (Marchesi & Andrews, 1971), chloroform/methanol (Hamaguchi & Cleve, 1972) and butanol (Anstee & Tanner, 1974) extractions. H.p.l.c. of extracted sialoglycoproteins on a gel permeation column (TSK G3000; 2.5 mm $\times$ 600 mm; LKB, Bromma, Sweden) was performed according to Blanchard *et al.* (1987).

RESULTS

Binding of the 12E7 antibody to human blood cells

Previous studies by Goodfellow (1983) have indicated that the 12E7 antibody reacts with a cell-surface antigen expressed on all human cells. On control experiments carried out by indirect immunofluorescence analysis however, we have consistently observed that the 12E7 determinant is present on human red cells, lymphocytes and platelets but not on granulocytes (results not shown). The 12E7 antigen is also early expressed, since it was

Table 1. Binding of the 12E7 monoclonal antibody to native and enzyme-treated red cells

Results are expressed as c.p.m. of iodinated anti-(mouse IgG) specifically bound to 5×10^7 red cells using a radioimmunoassay performed according to Casanova-Bettane *et al.* (1981). Briefly, 5×10^7 erythrocytes were incubated for 1 h at room temperature with 1:500 diluted ascitic fluid. Red cells were washed four times in 0.01 M-phosphate/0.15 M-NaCl, pH 7.5, containing 0.4% bovine serum albumin and 0.2% NaN_3 and further incubated with ^{125}I -labelled goat anti-(mouse IgG) (5×10^4 c.p.m.) in order to reveal the binding of the 12E7 monoclonal antibody.

Red cells	Treatment	12E7 antibody bound (c.p.m.)
Xg(a+) male	None	2812
Xg(a-) male	None	2076
Xg(a-) male	None	536
Xg(a+) female	None	2704
Xg(a-) female	None	539
Xg(a+) male	Papain	291
Xg(a+) male	Pronase	373
Xg(a+) male	Trypsin	352
Xg(a+) male	Chymotrypsin	173
Xg(a+) male	Neuraminidase	3042

easily detected on fetal red cells at 21 and 29 weeks of gestation (results not shown).

It is also known that the level of 12E7 antigen expression on red cells is determined by a quantitative polymorphism regulated by the Xg blood group (Goodfellow & Tippett, 1981). All Xg(a+) individuals have high expression of 12E7 whereas all Xg(a-) females are low level expressors. Xg(a-) males may have high or low expression of 12E7. Preliminary study of the 12E7 monoclonal antibody in a radiobinding assay using a ^{125}I -labelled rabbit anti-(mouse immunoglobulin) (Casanova-Bettane *et al.*, 1981) confirmed previous observations that high and low expressors of 12E7 antigen can be distinguished among human red cells (Table 1). Interestingly, the antigen was destroyed by prior treatment of intact red cells by several proteolytic enzymes, including papain, Pronase, trypsin and chymotrypsin, whereas no alteration could be demonstrated following hydrolysis by the neuraminidase from *Vibrio cholerae*. These results suggest that the 12E7 antigen is a protease-sensitive membrane component which presumably is carried by a cell surface protein or glycoprotein. Lymphocytes and platelets treated with proteases also lose the 12E7 antigen (results not shown).

The binding properties of the 12E7 monoclonal antibody to human blood cells was further investigated after ^{125}I -labelling of the purified IgG fraction. The antibody retained its biological activity after labelling with iodine and was used in order to determine the number of 12E7 receptors on blood cells. The data obtained at a wide range of antibody concentration (0.02–10 μg) were plotted according to Scatchard (1949) (Fig. 1). The number of binding sites was estimated as 1000/erythrocyte for a high-level expressor and as low as 100/erythrocyte for a low-level expressor of 12E7. Similarly, the number of antigen sites was estimated on different blood cells isolated from donors who were

Fig. 1. Binding equilibrium of the 12E7 monoclonal antibody to erythrocytes, platelets and lymphocytes

Erythrocytes (5×10^7) (high and low expressor of 12E7), 5×10^6 platelets and 5×10^5 lymphocytes were incubated for 1 h at room temperature with 0.02–10 μg of ^{125}I -labelled 12E7 antibody in 10 mM-phosphate buffer, pH 6.8, containing 150 mM-NaCl and 0.4% (w/v) bovine serum albumin (total volume 400 μl). The radioactivity specifically bound to the human blood cells was measured as described under 'Materials and methods' and the results were plotted according to Scatchard (1949). ●, ○, Red cells: high expressor (●) and low expressor (○); ■, lymphocytes + monocytes; ▲, platelets. Inset: saturation curves obtained with the same cells.

defined as high-level expressors of 12E7 on red blood cells. About 4000 antigen sites were found on platelets and 27000 on lymphocyte preparations (which contained some monocytes). No specific binding could be detected to human granulocytes. From the linear curves of Fig. 1 the binding constants calculated at equilibrium were $4 \times 10^7 \text{ M}^{-1}$ for red cells and platelets and $9 \times 10^7 \text{ M}^{-1}$ for lymphocytes respectively.

Identification and characterization of the 12E7 receptor of blood cells

In order to identify the blood cell component(s) which carry the 12E7 antigen the membrane proteins were analysed by the Western blot procedure. The 12E7 monoclonal antibody bound to a 28 kDa component on lymphocytes, red cells (Fig. 2a, lanes 2 and 3, respectively) as well as on native platelets (Fig. 2c, lane 3). An additional band of 25 kDa was heavily labelled on platelet and lymphocyte lysates, whereas it was barely detectable on red cell membranes. In agreement with the results from immunofluorescence discussed above, no

Fig. 2. Immunostaining of human red cell, platelet, lymphocyte and polymorphonuclear cell glycoproteins by the 12E7 monoclonal antibody after electrophoresis and transfer to nitrocellulose sheets

Membranes (150 μ g of protein) were solubilized in SDS buffer and submitted to electrophoresis in a 10% (w/v) polyacrylamide slab gel in the discontinuous system described by Laemmli (1970). Immediately after electrophoresis the proteins were transferred to nitrocellulose sheets (the transfer occurred in 20 mM-Tris/HCl/150 mM-glycine, pH 8.3, for 4 h at 30V constant voltage) which were then saturated with buffer containing 0.6% (w/v) gelatin and incubated overnight with a 1:100 dilution of the monoclonal antibody 12E7 (ascitic fluid). After extensive washings, the bound antibody was visualized by a further incubation with a peroxidase-labelled goat anti-immunoglobulin (diluted 1:200) followed by reaction with diaminobenzidine in the presence of H_2O_2 . The nitrocellulose was then dried on filter paper and exposed to Cronex 4 X-ray film for 3 days (a). Lane 1, control membranes from 3H -labelled erythrocyte membrane glycoproteins (2×10^5 c.p.m.); lane 2, lymphocytes; lane 3, red cells; lane 4; polymorphonuclear cells. (b) Immunostaining obtained after prior treatment of human red cells with trypsin (lane 1) and chymotrypsin (lane 2). (c) Immunostaining obtained after prior treatment of human platelets with trypsin (lane 1) and chymotrypsin (lane 2). Lane 3 is a control from untreated platelets and lane 4 shows the pattern obtained with the cytosolic fraction of platelet preparation (see under 'Materials and methods'). Nomenclature of glycoproteins is after Anstee *et al.* (1979).

Fig. 3. Presence of the 12E7 antigen on membrane glycoproteins from rare erythrocyte variants and effect of neuraminidase treatment on 12E7 antigenic expression

SDS/polyacrylamide-gel electrophoresis of membrane proteins and transfer to nitrocellulose sheets were performed as described for Fig. 2. The gels were loaded with 150 μ g of membrane proteins from red cells and platelets. (a) Immunostaining of red cell membrane glycoproteins by the 12E7 monoclonal antibody as described in Fig. 2. Lane 1, control donor of common phenotype; lane 2, En(a-); lane 3, S-s-U-; lane 4, Ge(-). (Immunodetection of glycoproteins from native and neuraminidase-treated platelets and red cells by the 12E7 monoclonal antibody. Lane 1, platelets treated with 0.1 unit of neuraminidase; lane 2, untreated platelets; lanes 3-6, red cells treated with 0.5, 0.1, 0.05 and 0.025 unit of neuraminidase, respectively; lane 7, untreated red cells.

Fig. 4. SDS/polyacrylamide-gel electrophoresis of the 12E7 preparation

Red cell membrane proteins (150 μ g) and the partially purified 12E7 preparation (3 μ g as sialic acid) were solubilized in SDS, loaded in a 11% (w/v) polyacrylamide gel and electrophorized in the discontinuous buffer of Laemmli (1970). The gel was stained by the periodate/Schiff procedure. Densitometric analysis was performed with a Vernon spectrophotometer equipped with a green filter (no. 57A). (a) Whole red cell membrane preparation; (b) 12E7 glycoprotein preparation. Nomenclature of glycoproteins is after Anstee *et al.* (1979).

binding could be detected in the cell extract prepared from polymorphonuclear cells (Fig. 2a, lane 4). In comparison with the mobility of red cell membrane sialoglycoproteins, the 28 and 25 kDa components migrate between glycophorin C (synonym: glycoprotein β or component D) and glycophorin D (synonym: glycoprotein γ or component E) (Fig. 2a, lane 1). This mobility was not affected by prior reduction of the samples with β -mercaptoethanol (results not shown).

Western blot analyses performed from trypsin and chymotrypsin-treated red cells confirm the loss of 12E7 antigen following proteolytic hydrolysis of intact cells, since no binding could be detected on membrane proteins from these cells separated by SDS/polyacrylamide-gel electrophoresis (Fig. 2b, lanes 1 and 2). Using protease-treated platelets, we found that the band at 28 kDa was no longer detectable, whereas the band at 25 kDa was not affected (Fig. 2c, lanes 1 and 2). These results suggest that the 12E7 monoclonal antibody might detect a cytosolic component not destroyed by proteolytic treatment of intact cells. Indeed, analysis of the cytosolic fraction from the platelet lysate prepared by the glycerol technique (Barber & Jamieson, 1970) shows the presence of a single band at 25 kDa (Fig. 2c, lane 4). It is likely

Fig. 5. Inhibition of human anti-Xg^a serum and 12E7 murine monoclonal antibody by red cell membrane glycoproteins and the partially purified 12E7 preparation

The partially purified 28 kDa component (see Fig. 4) was used for inhibition of the anti-Xg^a ($\blacktriangle$) and of the 12E7 monoclonal ($\triangle$) antibodies. In the inhibition test, up to 2 μ g (as sialic acids) of purified material (100 μ l) was first incubated with 100 μ l of human anti-Xg^a serum or 12E7 ascite fluid diluted 1:2 or 1:1500 respectively. After 1 h at room temperature, 5×10^7 Xg(a+) red cells (strong expressors of 12E7) were added and the reaction mixture was further incubated for 1 h at room temperature. The antibody specifically bound was measured as described in Table 1, except that 125 I-labelled protein A (5×10^4 c.p.m.) was used to estimate the binding of the human antibody. For comparison, glycophorins A ($\blacksquare$), B (+) and C ($\bullet$) were tested for inhibition of the anti-Xg^a serum.

that the band at 25 kDa from lymphocyte and red cell lysates (Fig. 2a, lanes 2 and 3) represents this cytosolic component which cross-reacts with the 12E7 antibody. This component has also been detected by Banting *et al.* (1985) on both human HEB7A and mouse LMTK cell lines, and is not presently considered as a degradation product of the 28 kDa polypeptide.

The presence of the 28 kDa component identified by the 12E7 monoclonal antibody was investigated also using rare variants red cells from En(a-), S-s-U- and Ge(-) individuals which lack glycophorin A, glycophorin B or glycophorins C and D, respectively (for review see Anstee, 1981; Dahr, 1986). The results shown in Fig. 3(a) indicate clearly that the 28 kDa component is normally present on these cells and further confirm that it is not a degradation product of a known sialoglycoprotein.

Further investigations have shown that the 28 kDa polypeptide is a sialoglycoprotein since it is affected by neuramidase. Indeed, following hydrolysis of intact platelets with 0.1 unit of neuramidase from *Vibrio cholerae*, the molecular mass of the 28 kDa band is reduced to 22 kDa whereas the 25 kDa component is not affected (Fig. 3b, lanes 1 and 2). Similarly the treatment of intact human red cells by increasing amounts of neuraminidase followed by electrophoresis of the membrane proteins and immunodetection by the 12E7 antibody resulted in the progressive disappearance of the 28 kDa component and simultaneous appearance of the 22 kDa band. These results strongly suggest that the 12E7 epitope is carried by a sialic-acid-containing glycoprotein, but its antigenic property is not dependent on this carbohydrate residue.

Fractionation of red cell membrane sialoglycoproteins and partial separation of the 28 kDa component from the known glycoproteins

Crude extracts of sialoglycoproteins were obtained as described under 'Materials and methods'. Analysis by Western blot of the crude preparations indicated that the 28 kDa component was extracted by the butanol or chloroform/methanol procedures, whereas it was no longer detectable after phenol/saline or Lis/phenol extractions (results not shown). This suggests that the 12E7 component behaves as a sialoglycoprotein but that the antigenicity of the protein is destroyed following exposure to phenol. After h.p.l.c. gel permeation chromatography on a G3000 TSK column of a crude sialoglycoprotein extract obtained by butanol partitioning, glycophorin A was separated from the 28 kDa component which co-eluted with glycophorins B and C (Blanchard *et al.*, 1987; results not shown). The protein was further purified on preparative SDS/polyacrylamide-gel electrophoresis using an 11% (w/v) gel. Electroelution of the 28 kDa protein from the gel was carried out in dialysis membranes at 4 °C in 30 mM-Tris/acetate buffer, pH 7.4, containing 0.1% SDS (150 mA, for 8 h). After concentration and dialysis on Centricon 10, the purity of the material was checked by SDS/polyacrylamide-gel electrophoresis.

As shown in Fig. 4, two major bands at 28 and 55 kDa were depicted following periodate/Schiff staining. The 55 kDa component was tentatively identified as the dimeric form of the 12E7 protein (28 kDa) since it bound the 12E7 monoclonal antibody after immunoblotting (results not shown). No additional band could be detected after Coomassie Blue staining. However, the 28 kDa component was not totally pure since a monoclonal antibody directed against glycophorin C (Rouger *et al.*, 1983) bound to this material blotted on a nitrocellulose sheet (results not shown). It is assumed that the 12E7 preparation contains a fragment of glycophorin C which was degraded at its C-terminus and which comigrate with the 28 kDa component (Blanchard *et al.*, 1987).

Biological activity of the partially purified material

The 12E7 preparation obtained from the preparative SDS/polyacrylamide gel was precipitated with 9 vol. of cold acetone (after addition of 50 µg of bovine serum albumin) in order to remove trace amounts of SDS and used in inhibition assays with the 12E7 monoclonal antibody and a human anti-Xg^a serum (Fig. 5). We found that 1 µg (as sialic acids) of this material inhibited

about 70% of the binding of anti-Xg^a or 12E7 antibodies to Xg(a+) red cells which are high expressors of 12E7. A second anti-Xg^a serum (gift from Miss P. Tippet, MRC Blood Group Unit, London) was also inhibited by this preparation (results not shown). Pure glycophorins A, B and C obtained according to Blanchard *et al.* (1987) did not inhibit at all over a large range of concentration, indicating that the inhibitory activity of the 12E7 preparation is not due to degradation fragment of a known sialoglycoprotein.

DISCUSSION

In this report we have investigated the binding properties of the 12E7 murine monoclonal antibody to human blood cells and attempted to purify the antigen from human red cells.

In agreement with previous studies (Goodfellow, 1983) the 12E7 monoclonal antibody binds to human blood cells and direct studies by Scatchard analyses using the ¹²⁵I-labelled antibody indicated that red cells, platelets and lymphocytes carried each approx. 1000, 4000 and 27000 antigen sites with binding constants ranging from 4×10^7 to 9×10^7 M⁻¹. However, we were unable to demonstrate a specific binding to human granulocytes, a finding which was confirmed by an indirect binding assay with a fluorescent anti-IgG probe as well as following Western blot analysis (see below). It is likely therefore that there is no receptor for the 12E7 antibody on human granulocytes, and this finding contradicts those from Goodfellow (1983).

Levy *et al.* (1979) and Banting *et al.* (1985) have shown that a 28–32.5 kDa component from human and mouse leukaemia cell lines could be immunoprecipitated or revealed by Western blot analysis using the 12E7 monoclonal antibody. In this report we have also found by Western blotting that the 12E7 antibody binds to a 28 kDa molecule which was revealed following incubation of red cell, platelet and lymphocyte proteins blotted to a nitrocellulose sheet. Again, we could not detect any component from granulocyte lysates. In addition, a component of 25 kDa apparent molecular mass was also identified mostly in platelet and lymphocyte lysates, but is a very minor component in red cell membranes. We have found that the 28 kDa component is protease-sensitive whereas the 25 kDa band is not, as shown when intact cells are pretreated with trypsin or chymotrypsin. In addition, prior treatment of intact cells with neuraminidase modifies the electrophoretic mobility of the 28 kDa component to an apparent molecular mass of 22 kDa, whereas again the 25 kDa band is not affected. These findings indicate that the 12E7 antibody binds to a sialic-acid-independent epitope carried by a protease-sensitive cell surface glycoprotein (28 kDa) and cross-reacts with a cytosolic component of 25 kDa. A similar cytoplasmic component has been also identified in the cytosol from human and mouse cell lines (Banting *et al.*, 1985) but the structural relationship between the cell-surface and cytoplasmic components has not yet been elucidated.

The glycoprotein of 28 kDa which is specifically recognized by the 12E7 monoclonal antibody was further characterized on red cells. Its electrophoretic mobility was intermediate between those of glycophorins C and D and it was present on variant red cells which lack either glycophorin A [En(a-)], glycophorin B (S-s-U-) or

both glycophorins C and D (Gerbich-negative cells). The presence of the 28 kDa component on all variant cells tested indicated that it is a unique molecule which does not derive by partial proteolysis from the known sialoglycoproteins. Therefore the 28 kDa glycoprotein is a new red cell membrane sialoglycoprotein whose properties are expected to be comparable with those of the extensively studied glycophorins. Indeed, it was extracted in the aqueous phase following chloroform/methanol/water (Hamaguchi & Cleve, 1972) and butanol/water (Anstee & Tanner, 1974) partitionings and could be partially separated from the major sialoglycoproteins by anion-exchange and gel-permeation chromatography.

The 28 kDa glycoprotein did not bind to the cytoskeleton since the bulk of the 12E7 activity is easily extracted from red cell membrane by low concentrations of non-ionic detergent (0.1 % Triton X-100) as judged by Western blot analysis of both supernatant and pellet. In this aspect, the 28 kDa component behaves like glycophorins A and B and is different from glycophorins C and D which are tightly bound to the cytoskeleton (Owens *et al.*, 1980; Dahr *et al.*, 1985).

Starting from Xg(a+) red cells which strongly express the 12E7 antigen (Table 1) we have obtained a partially purified preparation of the 28 kDa glycoprotein by a combination of butanol extraction, gel permeation chromatography and preparative SDS/polyacrylamide-gel electrophoresis procedures. This material, which was still contaminated by a small amount of glycophorin C degradation product, was used to inhibit a human anti-Xg^a serum and the 12E7 monoclonal antibody. Native glycophorins A, B and C used as controls were not inhibitory in these systems. We have found, however, that the 12E7 preparation inhibited both antibodies to a similar extent, suggesting either that the 12E7 and the Xg^a antigen copurified or that the same glycoprotein might carry both determinants. This is interesting, since previous studies have shown that the Xg^a antigen is also a protease-sensitive component of the red cell membrane (Habibi *et al.*, 1979). Recent investigations of the expression of Xg^a and 12E7 on primate red cells have indicated that both Xg(a+) and Xg(a-) gibbon erythrocytes failed to express high levels of 12E7 antigen, suggesting that Xg^a and 12E7 are different epitopes, but it is not established whether these antigens are present on the same molecule (Shaw *et al.*, 1985).

It is likely that cloning of the DNA sequences corresponding to the *MIC2* locus will provide rapidly the complete molecular structure of the 12E7 glycoprotein (Darling *et al.*, 1986). Indeed, study of a cDNA clone derived from the *MIC2* gene already indicates some properties of the 12E7 antigen, which is a transmembrane glycoprotein with extracellular orientation of its *N*-terminus. The exposed domain carrying the 12E7 antigen lacks *N*-linked glycosylation sites but contains several potential sites for *O*-linked glycosylation (P. N. Goodfellow, personal communication). Obviously parallel knowledge of the protein structure carrying the 12E7 as well as Xg^a determinants will contribute to clarify the relationship between these antigens, and whether both determinants are carried by the same molecule.

gift of 12E7 monoclonal antibody, and to Miss P. Tippet (MRC Blood Group Unit, University College London) for the supply of human anti-Xg^a serum (And.).

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