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The In^a and In^b blood group antigens are located on a glycoprotein of 80,000 MW (the CDw44 glycoprotein) whose expression is influenced by the *In(Lu)* gene

F. A. SPRING, R. DALCHAU,* G. L. DANIELS,† G. MALLINSON, P. A. JUDSON, S. F. PARSONS, J. W. FABRE* & D. J. ANSTEE *South Western Regional Blood Transfusion Centre, Southmead, Bristol, *Blond McIndoe Centre for Medical Research, Queen Victoria Hospital, East Grinstead, Sussex and †MRC Blood Group Unit, University College London, London*

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SUMMARY

The In^a and In^b blood group antigens were found to be located on an erythrocyte membrane glycoprotein of 80,000 MW by immunoblotting with human anti-In^a and anti-In^b antibodies under non-reducing conditions. This glycoprotein is shown here to be identical to that defined by monoclonal antibodies to CDw44, and a new murine monoclonal antibody (BRIC 35) is added to this cluster. Experiments with endo-β-galactosidase and Endo F preparations suggest that the glycoprotein contains one or more *N*-glycans but that these oligosaccharides do not contain extensive poly-*N*-acetyl lacto saminyl sequences. Experiments using membranes prepared from sialidase-treated normal erythrocytes, from Tn erythrocytes and from Cad erythrocytes suggest that the glycoprotein does not contain a substantial content of *O*-glycans. The In^b antigen and the epitope defined by a murine monoclonal antibody (BRIC 35) show reduced expression on Lu(a-b-) erythrocytes which result from the effect of the dominant inhibitor gene *In(Lu)*. Evidence is presented here that the In^b antigen is expressed on normal granulocytes and lymphocytes and on the haemopoietic cell lines HEL, K562 and HL-60, a lymphoblastoid cell line and lymphocytes from two patients with B-CLL.

INTRODUCTION

There is increasing interest in cell surface glycoprotein molecules which are expressed on a broad range of cells and tissues. One such molecule, identified by several monoclonal antibodies and variously described as human brain–granulocyte–T-lymphocyte antigen (Dalchau, Kirkley & Fabre, 1980; McKenzie, Dalchau & Fabre, 1982), p80 glycoprotein (Telen, Eisenbarth & Haynes, 1983; Telen, Palker & Haynes, 1984) and p85 glycoprotein (Letarte, Iturbe & Quackenbush, 1985), is known to be expressed on a broad range of haemopoietic cells as well as on brain, heart, liver, thymus, kidney and breast and colon epithelium (Dalchau *et al.*, 1980; Daar & Fabre, 1981, 1983; Haynes *et al.*, 1983; Quackenbush *et al.*, 1986). Monoclonal antibodies which react with this glycoprotein define the cluster CDw44 (Cobbold, Hale & Waldman, 1987). In brain, the glycoprotein is preferentially localized in white matter (McKenzie *et al.*, 1982; Quackenbush *et al.*, 1985). Elevated levels of this glycoprotein in white matter from individuals aged between 55 and 80 and from brain of multiple sclerosis victims have been reported recently by Cruz *et al.* (1985, 1986). In the thymus, the glycoprotein is confined to the medullary compartment and is acquired by thymocytes during intrathymic T-cell maturation (Dalchau *et al.*, 1980; Haynes *et al.*, 1983; Quackenbush & Letarte, 1985).

Jalkanen *et al.* (1986) have suggested that this glycoprotein may be related to a lymphocyte glycoprotein involved in endothelial cell recognition and lymphocyte homing. The expression of this glycoprotein on erythrocytes may be influenced by the action of the dominant inhibitor gene *In(Lu)* (Telen *et al.*, 1984). The action of this gene, which is independent of the *Lutheran* locus, causes suppression of all Lutheran antigens. *In(Lu)* also suppresses several high frequency erythrocyte antigens which are not necessarily genetically controlled by the Lutheran locus (Daniels *et al.*, 1986).

In this paper, we describe a murine monoclonal antibody, BRIC 35, which recognizes the same glycoprotein as antibodies

Abbreviations: AET, 2-aminoethylisothiouronium bromide; BSA, bovine serum albumin; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia; CMML, chronic myelomonocytic leukaemia; DOC, sodium deoxycholate; DTT, dithiothreitol; IAA, iodoacetic acid; PMSF, phenylmethylsulphonyl fluoride; SDS-PAGE, SDS polyacrylamide gel electrophoresis.

Correspondence: Dr F. A. Spring, South Western Regional Blood Transfusion Centre, Southmead, Bristol BS10 5ND, U.K.

to CDw44. The reactivity of BRIC 35 with erythrocytes is dramatically affected by the action of the *In(Lu)* gene. We also show that the human blood group antigens, In^a and In^b, are both carried on this glycoprotein and demonstrate that the expression of In^b on erythrocytes is affected by the *In(Lu)* gene. The In^a antigen occurs in 2–4% of the Indian population (Badakere, Parab & Bhatia, 1974). Evidence for the occurrence of the antithetical antigen, In^b, was first reported by Giles (1975). We further show that the In^b antigen is not confined to erythrocytes but occurs on other peripheral blood cells and haemopoietic cells.

MATERIALS AND METHODS

Murine monoclonal antibody NBTS/BRIC 35 (BRIC 35, class IgG1) was cloned from a fusion performed using spleen cells from a BALB/c mouse given seven i.p. injections of human erythrocytes over a period of 7 weeks, as described by Spring *et al.* (1987).

Human anti-In^b serum (donor P.R.), In(b−) red cells [donor, P.R. In(a+b−)] and red cells from donor L.P. In(a+b+) were kindly supplied by Dr A. Prentice and Mr S. Davies, Blood Bank, Derriford Hospital, Plymouth, Devon. Human anti-In^b serum (Naj.) was a gift from Mr L. Dodson, Lewisham Hospital, London. Human anti-In^a serum (Mari.) was from Ms J. Poole, Blood Group Reference Laboratory, Oxford, Oxon. Normal erythrocyte samples were available from the Regional Transfusion Centre, Bristol. Human anti-In^b and human anti-In^a were purified for immunoblotting as described elsewhere (Tanner *et al.*, 1988). Erythrocytes from donor L.B. [recessive form of Lu(a−b−)] were obtained by Dr D. A. Webb of the Regional Transfusion Centre, Bristol, and those of Mor II-6 [X-linked type of Lu(a−b−)] by Mr P. Norman, Australian Red Cross Society, Adelaide, Australia. Lu(a−b−) erythrocytes of the *In(Lu)* type were kindly provided by Mrs M. Leak, South London Transfusion Centre, Tooting. Tn erythrocytes (donor Ba.) were kindly provided by Dr D. Lee and Dr C. Hodson, Lancaster Blood Transfusion Centre, Lancaster. Cad erythrocytes were kindly provided by Dr J. P. Cartron, Inserm Unite, Centre National de Transfusion Sanguine, Paris, France. The production and use of endo-β-galactosidase (*Flavobacterium keratolyticus*) and Endo F preparations (*F. meningospeticum*) was as described elsewhere (Tanner *et al.*, 1988), as are the methods for treatment of erythrocytes with trypsin, pronase, sialidase (Mallinson *et al.*, 1986) and chymotrypsin (Judson & Anstee, 1977). Treatment of erythrocytes with DTT and AET was as described elsewhere (Branch *et al.*, 1985; Advani *et al.*, 1982, respectively).

Normal peripheral blood leucocytes and platelets were prepared as described elsewhere (Judson *et al.*, 1983). Cell lines were grown as described previously (Spring *et al.*, 1987). Peripheral blood samples from patients with leukaemia were kindly provided by Dr P. Taylor, Bristol Royal Infirmary, Bristol. Transformation of peripheral blood lymphocytes with Epstein–Barr virus and subsequent culture of the cell lines was as described by Doyle *et al.* (1985), except that incubation of lymphocytes with virus (culture supernatant from a B95-8D marmoset cell line) was for 1 hr at 37°. Indirect immunofluorescence was either measured on paraformaldehyde-fixed leucocytes as described elsewhere (Spring *et al.*, 1987) or measured by flow cytometry with an EPICS C (Coulter Electronics Ltd, Luton, Beds), following the method of Loken & Stall (1982), omitting the propidium iodide staining.

Erythrocyte ghosts were prepared according to the method of Dodge, Mitchell & Hanahan (1963). Erythrocyte skeletons were prepared from erythrocyte ghosts by the method of Mueller & Morrison (1981), except that DTT was omitted from the extraction buffer. Skeletons were prepared using different concentrations of Triton X-100 (1%, 5% and 10%). Membranes from K562, HEL, HL-60 cells and from the mononuclear cells of two patients with B-CLL were prepared as described elsewhere (Spring *et al.*, 1987). SDS–polyacrylamide gel electrophoresis of erythrocyte ghosts was carried out under non-reducing conditions on gels containing either 10% or 8% acrylamide with a 3% overlay, using the method of Laemmli (1970). Immunoblotting was as described by Mallinson *et al.*, (1986), except that 5% (w/v) bovine milk powder was used as the blocking agent. Immunoprecipitation was carried out using BRIC 35 coupled to Sepharose 4B. BRIC 35 (3·5 mg), purified on a rabbit anti-mouse IgG column as described elsewhere (Merry *et al.*, 1986), was coupled to CNBr-activated Sepharose 4B (300 mg; Pharmacia Ltd, Milton Keynes, Bucks) according to the manufacturer's instructions. Erythrocyte ghosts (2 ml) were solubilized (15 min at 23°) in four volumes PBS, pH 7·2, containing 1% Triton X-100 and 2 mM PMSF, a 15% (v/v) suspension of BRIC 35-coated Sepharose beads (3 ml) in the same buffer added, and the whole incubated for 3 hr at 23° with gentle mixing. The beads were washed four times in PBS pH 7·2/1% Triton X-100/2 mM PMSF before addition of sample buffer (20 mM Tris-HCL pH8·0/5 mM EDTA/5% SDS/10% glycerol/0·1 mg/ml bromophenol blue/0·1 mg/ml Pyronin Y/2 mM PMSF). The supernatant was recovered and stored frozen (−40°) prior to SDS–polyacrylamide gel electrophoresis.

F10.44.2 antigen was prepared from approximately 2×10^{11} T-CLL cells (kindly provided by Dr E. Dewar, Department of Pathology, Edinburgh, from a patient undergoing therapeutic leucophoresis), using a combination of monoclonal antibody-affinity, ion-exchange, lectin-affinity and gel-permeation chromatography techniques. The washed T-CLL cells were solubilized in 5% Brij 96:Brij 99 (mixed in a 1:2 ratio) containing proteolytic inhibitors, and its purification carried out as previously described in detail (Dalchau & Fabre, 1982).

Solid-phase indirect ^{125}I-binding assays were performed, using purified F10.44.2 antigen as the target, to study the specificity of the BRIC 35 and anti-In^b antibodies. Twenty-five microlitres of pure F10.44.2 antigen were incubated in PVC microtitre plates overnight at 4°. The antigen was removed, then 100 μl 5% bovine serum albumin (BSA) in PBS was added to each well and incubated for 30 min at 4°. This was removed and the wells were washed three times with 0·1% BSA/PBS. Twenty-five microlitres of a saturating concentration of the BRIC 35 and F10.44.2 mouse monoclonal antibodies, and neat, purified anti-In^b and purified anti-D human antibodies, and 0·5% BSA/PBS as a control, were added to the wells and incubated for 1 hr at 4°. The antibodies were removed from the wells which were washed three times with 0·1% BSA/PBS. Fifty microlitres of ^{125}I-labelled immunoadsorbent-purified rabbit $F(ab')_2$ anti-mouse

Table 1. Reduced expression of the In[b] antigen on Lu (a−b−) erythrocytes of the *In(Lu)* type

Family member	Antibody				
	Lu[a]	Lu[b]	Lu[3]	BRIC 35	In[b]
I-1	0	0	0	0	7
I-2	0	+	+	+	41
I-3	0	+	+	+	22
II-1	0	0	0	0	0
II-2	0	+	+	+	21
II-3	0	+	+	+	28

Figures for In[b] represent agglutination titration scores. (+) Indicates agglutination of erythrocytes; (o) indicates lack of agglutination.

$F(ab')_2$ or 50 μl ^{125}I-labelled immunoadsorbent-purified rabbit $F(ab')_2$ anti-human $F(ab')_2$ containing 300,000 c.p.m. (Dalchau & Fabre, 1979) were added to the wells as appropriate, incubated for 1 hr at 4°, then washed four times. The wells were cut out and counted in a gamma counter.

RESULTS

The specificity of BRIC 35 on human erythrocytes

Monoclonal antibody BRIC 35 agglutinated normal human erythrocytes and sialidase-treated normal erythrocytes, but not erythrocytes treated with trypsin, chymotrypsin, pronase, AET or DTT. The epitope recognized by this antibody was markedly susceptible to treatment with DTT, since concentrations as low as 50 mM DTT effected complete loss of reactivity with the antibody and the reactivity was substantially reduced at 10 mM.

When BRIC 35 was tested with an extensive range of erythrocytes with rare blood group phenotypes lacking high frequency blood group antigens, including In(a+b−), the only erythrocytes that gave weakened or negative reactions were those from individuals of the Lu(a−b−) phenotype resulting from the action of the dominant inhibitor gene *In(Lu)* (five individuals from four different kindred were used; data for one kindred is shown in Table 1). Lu(a−b−) erythrocytes resulting from the recessive form of inheritance (Darnborough *et al.*, 1963) or the X-linked form of inheritance (Norman, Tippett & Beale, 1986) gave normal reactions with BRIC 35 (data not shown).

The erythrocyte component(s) with which BRIC 35 reacts was identified by immunoblotting under non-reducing conditions. No reactive bands were obtained when membrane samples were analysed under reducing conditions (5% 2-mercaptoethanol). Immunoblots of normal erythrocyte membranes from 8% acrylamide gels revealed a major band of 85,000±2100 MW ($n=4$, Fig. 1a, denoted the 85,000 component). In contrast, immunoblots of normal erythrocyte membranes separated in 10% acrylamide gels gave a major band of 78,750±1400 MW ($n=7$, Fig. 1c, denoted the 80,000 component). The MW values obtained for a given acrylamide gel concentration were reproducible and were consistently different for the two acrylamide concentrations. Weak or negative agglutination reactions of BRIC 35 with Lu(a−b−) erythrocytes of the *In(Lu)* type were confirmed by immunoblotting (Fig. 1e), whilst membranes derived from Lu(a−b−) erythrocytes of the recessive type (Fig. 1d) and the X-linked types (Fig. 1g) gave reactions comparable to those of normal erythrocytes.

When immunoblots were carried out on membranes derived from normal erythrocytes treated with an Endo F preparation, a major band of 75,500 MW was observed on immunoblots from 8% gels ($n=2$, Fig. 1b). This represents a reduction of 9700 MW in comparison with normal membranes and suggests that the molecule is a glycoprotein containing one or more *N*-glycans. When comparable immunoblots were carried out using 10% acrylamide gels, the reduction observed was 6350 ($n=2$, data not shown). Immunoblotting of membranes from endo-β-galactosidase-treated normal erythrocytes did not demonstrate any differences in comparison with normal membranes (data not shown), suggesting that the *N*-glycan(s) present on this molecule do not contain extensive poly-*N*-acetyllactosaminyl groups. A normal pattern of reactivity was also observed when membranes from sialidase-treated normal erythrocytes and from the erythrocytes of a patient with Tn syndrome were examined (data not shown). These results suggest that the BRIC 35-reactive component does not contain a substantial content of *O*-glycans.

Identification of the human erythrocyte glycoprotein carrying the In[a] and In[b] antigens

Immunoblots of In(b+) human erythrocyte membranes using two purified human anti-In[b] preparations (prepared as described in the Materials and Methods) demonstrated the presence of a major band with a leading edge of 77,400±1600 MW ($n=8$) and a trailing edge of 80,750±2100 MW ($n=8$) under non-reducing conditions in 10% acrylamide gels (Fig. 2a). This band (denoted the 80,000 component) was absent from In(b−) erythrocyte membranes (Fig. 2b). A minor band with a leading edge of 68,500±1700 ($n=4$, denoted the 70,000 component) was also apparent on immunoblots of In(b+) membranes (Fig. 2a). Minor components of greater MW than the 80,000 component were also apparent on In(b+) membranes, but absent on In(b−) membranes (Fig. 2a,b). Binding of anti-In[b] to both the 80,000 component and the 70,000 component was markedly reduced when In(b+) membranes from either trypsin-treated or chymotrypsin-treated erythrocytes were used, whilst no binding to In(b+) membranes from pronase-treated erythrocytes was observed (data not shown). These In[b] reactive components consistently gave higher MW values on 8% acrylamide gels. The major component (denoted the 85,000 component) had a leading edge of 81,500 and a trailing edge of 85,000 (mean of two determinations), while the minor component (denoted the 75,000 component) had a leading edge of 73,500 MW and a trailing edge of 76,000 (mean of two determinations; Fig. 2i).

When immunoblots were carried out with membranes from In(b+) erythrocytes treated with Endo F preparations, new bands of 78,000 MW (mean of two determinations) and 68,500 MW (mean of two determinations) were apparent on 8% gels (Fig. 2j). More stringent treatment with Endo F resulted in the disappearance of the 85,000 MW band completely (data not shown). Treatment of In(b+) erythrocytes with endo-β-galac-

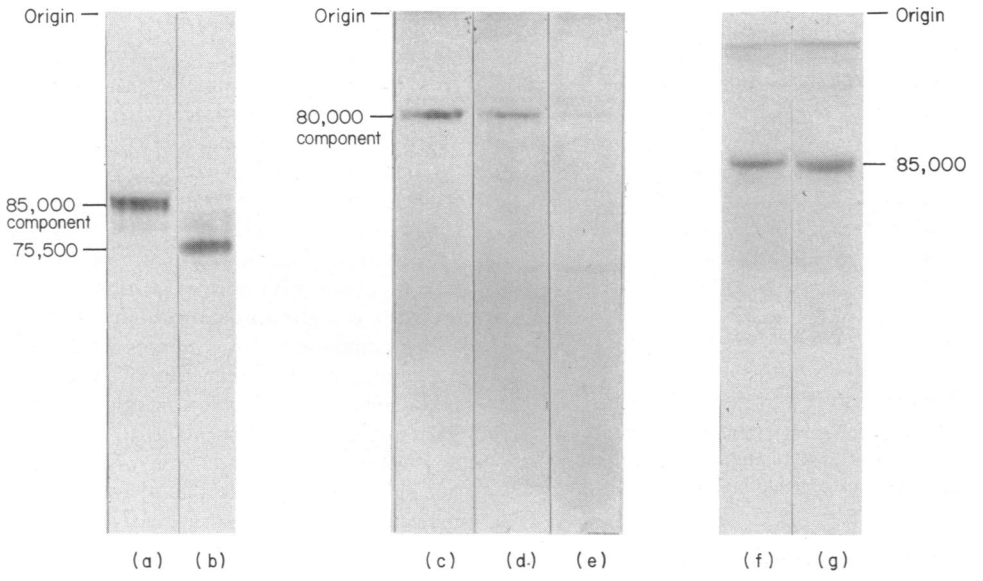

Figure 1. Identification of BRIC 35-reactive components in human erythrocyte membranes by immunoblotting. Erythrocyte membranes were solubilized under non-reducing conditions and immunoblotted with mouse monoclonal antibody BRIC 35. Tracks (a), (b), (f) and (g) were run on acrylamide gels containing 8% (w/v) acrylamide in the separating gel; tracks (c)–(e), were run on an acrylamide gel containing 10% (w/v) acrylamide in separating gel; tracks (a) and (b) were from the same experiment; (a) normal erythrocyte ghosts; (b) ghosts from erythrocytes treated with an Endo F preparation; tracks (c)–(e) were from the same experiment; (c) normal erythrocyte ghosts; (d) erythrocytes ghosts from an Lu(a−b−) donor of the recessive type (donor L.B.); (e) erythrocyte ghosts from an Lu(a−b−) donor of the *In(Lu)* type (donor Sheff.); tracks (f) and (g) were from the same experiment; (f) normal erythrocytes; (g) erythrocytes from an Lu(a−b−) donor of the X-linked type (donor Mor. II-6).

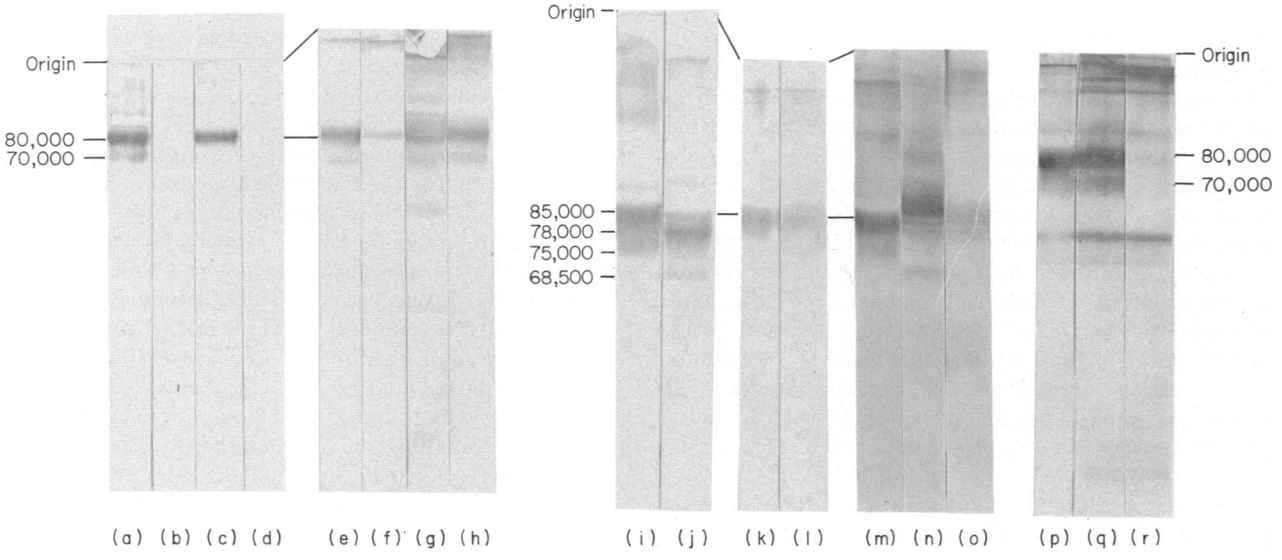

Figure 2. The glycoprotein components that carry In^a and In^b blood group activity of erythrocyte membranes and those of leucocyte membranes that carry In^b blood group activity. Membranes were solubilized under non-reducing conditions. Tracks (a), (b) and (e)–(r) were blotted with anti-In^b whilst tracks (c) and (d) were blotted with anti-In^a. Tracks (a)–(h) and (p)–(r) were run on 10% acrylamide gels whilst tracks (i)–(o) were run on 8% acrylamide gels. Tracks (a)–(d) were from the same experiment where (a) and (b) were incubated with anti-In^b and (c) and (d) were incubated with anti-In^a; (a) and (d) normal erythrocytes; (b) and (c) In(b−) erythrocytes (donor P.R.); tracks (e)–(h) were from a second experiment; (e) normal erythrocytes; (f) Lu(a−b−) erythrocytes of the *In(Lu)* type (donor Sheff.); (g) Lu(a−b−) erythrocytes of the X-linked type (donor Mor.II-6); (h) Lu(a−b−) erythrocytes of the recessive type (donor L.B.); tracks (i) and (j) were from the same experiment; (i) normal erythrocytes; (j) normal erythrocytes treated with an endo F preparation; tracks (k) and (l) were from the same experiment; (k) normal erythrocytes; (l) cytoskeletons prepared with 10% Triton X-100; tracks (m)–(o) were from the same experiment; (m) normal erythrocytes; (n) membranes from HEL cells; (o) membranes from K562 cells; tracks (p)–(r) were from the same experiment; (p) normal erythrocytes; (q) BRIC 35 immunoprecipitated material from normal erythrocytes; (r) BRIC 35 immunoprecipitated material from pronase-treated erythrocytes.

tosidase did not result in any apparent change in the MW of either the major or minor components (data not shown). Sialidase-treated In(b+) erythrocytes, erythrocytes from an individual with Tn syndrome and erythrocytes from the original Cad-positive propositus (Cazal *et al.*, 1968) showed a normal pattern of anti-In^b binding (data not shown). Immunoblots of red cell skeletons prepared from normal ghosts by extraction with either 1%, 5% (data not shown) or 10% Triton X-100 (Fig. 2l) demonstrated the presence of the major In^b-reactive glycoprotein component.

Immunoblots of membranes from In(a+b−) and In(a−b+) erythrocytes using human anti-In^a demonstrated that the In^a antigen is located on a component of the same MW as that recognized by anti-In^b (Fig. 2a–d). Membranes prepared from In(a+) and In(b+) erythrocytes treated with sialidase, pronase, trypsin, chymotrypsin, endo-β-galactosidase and Endo F and immunoblotted with anti-In^a and anti-In^b, did not demonstrate differences between the In^a- and In^b-reactive components (data not shown).

The expression of the In^b antigen is affected by the *In(Lu)* gene

The expression of the In^b antigen on some Lu(a−b−) erythrocytes is weaker than that on Lu(a−b+) erythrocytes. Table 1 shows the results of titrating anti-In^b with the erythrocytes of members of a family in which the inheritance of the Lu(a−b−) phenotype, due to the dominant inhibitor *In(Lu)* gene, occurs in two individuals. Titrations of anti-In^b with normal erythrocytes and with Lu(a−b−) erythrocytes of both the recessive type and the X-linked type show that the expression of In^b is not depressed in these phenotypes (data not shown).

Erythrocyte membranes from various Lu(a−b−) donors were prepared and examined for their reactivity with anti-In^b by immunoblotting. The pattern of binding to membranes from the recessive and X-linked Lu(a−b−) donors was similar to that seen for normal membranes (Fig. 2g,h), whilst membranes from Lu(a−b−) donors of the *In(Lu)* type showed reduced binding of anti-In^b (Fig. 2f) when compared to normal membranes (Fig. 2e).

The presence of the BRIC 35 epitope and the In^b antigen on peripheral blood leucocytes and haemopoietic cell lines

All peripheral blood leucocytes (granulocytes, T lymphocytes and the mononuclear cells remaining after T lymphocyte depletion) but not platelets, gave strong immunofluorescence with BRIC 35. BRIC 35 also gave strong immunofluorescence with the erythroleukaemic cell line HEL, the promomyelocytic line HL-60 and the B lymphoblastoid line IM-9, and weaker immunofluorescence with the T-ALL cell line MOLT 4. In contrast to these cell lines, where over 90% of the cells were reactive with BRIC 35, only 25% of cells of a second erythroleukaemic cell line, K562, were positive. Negative reactions were obtained with two Burkitt's lymphoma lines (Raji and Daudi). Leucocytes isolated from the peripheral blood of patients with different types of chronic leukaemias also gave positive reactions with BRIC 35. Thus, in patients with B-CLL (eight cases), and a patient with CMML, greater than 90% of the cells were positive, whereas in a case of T-CLL and in patients with CML (five patients), approximately 80% of the cells were positive.

EBV-transformed lymphoblastoid lines from the recessive Lu(a−b−) type (donor L.B.) and the dominant Lu(a−b−) type (donor Els.) were examined for reactivity with BRIC 35. Both cell lines were strongly reactive with BRIC 35 and these reactions did not appear different from those obtained with cell lines from presumptive Lu(b+) donors (data not shown).

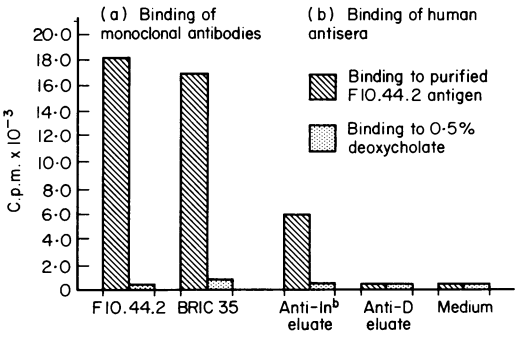

Figure 3. Binding of BRIC 35 and human anti-In^b to purified F10.44.2 antigen. (a) Binding of monoclonal antibodies, F10.44.2 and BRIC 35; (b) binding of purified human anti-In^b and purified human anti-D.

Human anti-In^b reacted by immunofluorescence with 85% of normal granulocytes and 80% of unfractionated mononuclear cells. When membranes from several leucocyte lines were examined by immunoblotting, human anti-In^b bound to a broadly migrating component on all the preparations examined. The MW of the reactive bands varied slightly on the different leucocytes tested. The major reactive components of HEL cells (Fig. 2n), K562 cells (Fig. 2o), lymphocytes from two patients with B-CLL (data not shown), an EBV-transformed line from a patient with Tn syndrome (donor Ba., data not shown) and HL-60 cells (data not shown) were similar in size to that of erythrocytes. The binding intensity of anti-In^b to K562 cells (Fig. 2o) was weaker when compared with that binding to HEL cells (Fig. 2n; comparable amounts of protein were applied for each cell type), and this, presumably, reflects the lower proportion of In^b-positive K562 cells compared with In^b-positive HEL cells, which was demonstrated by the binding of BRIC 35 in immunofluorescence tests.

Murine monoclonal antibody BRIC 35 and human anti-In^b react with the same glycoprotein in erythrocytes and leucocytes

BRIC 35 was used to prepare immunoprecipitates from normal and pronase-treated erythrocyte membranes (see the Materials and Methods), and immunoblotted with human anti-In^b. The results clearly demonstrate that these antibodies react with the same glycoprotein in normal erythrocytes (Fig. 2p–r). BRIC 35 does not, however, have anti-In^b specificity since it reacts with the erythrocytes of an In(b−) donor (data not shown).

BRIC 35 showed a similar pattern of reactivity with normal leucocytes and haemopoietic cell lines to that obtained with antibodies to CDw44 (Cobbold *et al.*, 1987). Antibody F10.44.2, one of the antibodies in this cluster, has been used to isolate the reactive glycoprotein from human leucocytes (McKenzie *et al.*, 1982). BRIC 35 and human anti-In^b were tested, therefore, for binding to this purified glycoprotein. The results clearly show that both BRIC 35 and human anti-In^b bind to the purified F10.44.2 glycoprotein (Fig. 3). Human anti-D

was used as a control for non-specific binding of human IgG and showed no significant binding (Fig. 3). These results show that BRIC 35 and human anti-In^b react with the same glycoprotein in leucocytes.

DISCUSSION

These results show that monoclonal antibody BRIC 35 and human anti-In^a and anti-In^b recognize different determinants on a surface glycoprotein of 80,000 MW in human erythrocytes. This glycoprotein is the same as that previously described by Dalchau *et al.* (1980), Telen *et al.* (1984) and Letarte *et al.* (1985) and is defined by antibodies in CDw44 (Cobbold *et al.*, 1987). The In^a and In^b antigens are the first blood group antigens to be assigned to the CDw44 glycoprotein. Our finding that anti-In^b reacts with a component on In(a−) membranes of the same MW as that which reacts with anti-In^a on In(b−) membranes is consistent with the suggestion of Giles (1975) that In^b is the antithetical antigen to In^a.

The CDw44 molecule is sensitive to treatment with pronase, trypsin and chymotrypsin on intact erythrocytes since such treatment markedly reduces reactivity with BRIC 35, anti-In^a and anti-In^b. The molecule is also very sensitive to reduction, suggesting that it has an essential requirement for inter- and/or intra-chain disulphide bonding. Letarte *et al.* (1985) have provided evidence that the glycoprotein consists of a single polypeptide chain with one or more intra-chain disulphide bonds. Our results suggest that at least a proportion of the CDw44 glycoprotein is associated with the red cell skeleton since material reactive with anti-In^b remains associated with the skeleton even after extraction with 10% Triton X-100. This result is of particular interest in view of the recent paper of Udden *et al.* (1987) who reported two families in which inheritance of the *In(Lu)* gene was associated with abnormalities of red cell morphology, since it raises the possibility that skeletal associations which influence normal red cell shape may involve the CDw44 protein.

Our data, obtained by immunoblotting of membranes from Endo F-treated erythrocytes with BRIC 35 and anti-In^a and anti-In^b indicate that the CDw44 glycoprotein contains one or more *N*-glycosidically linked oligosaccharides. These results are in agreement with the work of Letarte *et al.* (1985) and Telen, Hala & Haynes (1986). These *N*-glycans do not appear to contain polylactosamine sequences since treatment of intact erythrocytes with endo-β-galactosidase had little effect on the MW of the glycoprotein. The determinants seen by BRIC 35 and human anti-In^a and anti-In^b and at least two epitopes on the glycoprotein defined by Telen & Letarte (1986) do not appear to be dependent on the presence of *N*-glycans. Immunoblotting of erythrocyte membranes from a donor with Tn syndrome and with membranes from the original Cad donor did not show any marked differences from normal, suggesting that the molecule does not have a substantial content of *O*-glycans, although it does not rule out their occurrence altogether. In fact, carbohydrate analyses on the intact purified molecule confirm the presence of both *N*-linked and *O*-linked sugars (B. F. Flanagan, personal communication).

We have observed that the MW of glycoprotein CDw44 varies on SDS–PAGE under non-reducing conditions according to the acrylamide concentration of the gel. This phenomenon is well known for glycoproteins (Leach, Collawn & Fish, 1980), but has not been reported previously for CDw44.

In^b and the antigen defined by BRIC 35 are both depressed on Lu(a−b−) erythrocytes of the *In(Lu)* type. Telen & Letarte (1986) reported that seven monoclonal antibodies, that define three different epitopes on the CDw44 glycoprotein, showed reduced binding (range 23–45% of normal) with Lu(a−b−) erythrocytes of the *In(Lu)* type. The *In(Lu)* gene causes a reduction in the reactivity of red cells with antibodies of the Lutheran blood group system and antibodies against several other common antigens which are not part of the Lutheran blood group system (summarized in Daniels *et al.*, 1986). The In^b antigen can now be added to the list of affected antigens. It has been suggested that these effects may result from abnormal glycosylation of a common carbohydrate sequence (Marcus, Kundu & Suzuki, 1981). However, if this is so, the glycoprotein does not appear to have an altered MW as is the case for sialoglycoproteins of Tn and Cad erythrocytes.

Our results indicate that the *In(Lu)* gene does not have a dramatic effect on the expression of the BRIC 35 epitope on lymphoblastoid cells derived by EB virus transformation of B lymphocytes from individuals whose Lu(a−b−) red cells are of the *In(Lu)* type. Telen *et al.* (1986) report that while the majority of peripheral blood lymphocytes from such donors appear normal with respect to expression of epitopes recognized by monoclonal antibodies to CDw44, approximately one-third of monocytes have deficient expression. Whether this deficiency is associated with any functional abnormality in these monocytes is unknown. However, the broad tissue distribution of CDw44 glycoprotein (Dalchau *et al.*, 1980; Haynes *et al.*, 1983; Quackenbush *et al.*, 1986) suggests that the molecule has an important general functional role at cell surfaces and investigation of its expression in haemopoietic cells from such Lu(a−b−) individuals may be a useful approach towards elucidating its function.

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