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The quantification of erythrocyte antigen sites with monoclonal antibodies

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Summary. The application of monoclonal antibodies to the quantification of blood group antigen sites on erythrocytes was examined. A second antibody technique using labelled anti-mouse IgG could not be used as it was not possible to predict the binding ratio between this and the monoclonal antibody. A series of monoclonal antibodies (R10, R18, BRIC 13, BRIC 14) to the erythrocyte sialoglycoprotein α (syn: glycophorin A) showed the number of antigen sites to be from $0{\cdot}3 \times 10^6$ to $1{\cdot}2 \times 10^6$ per erythrocyte and supported the conclusion that the Wr^b antigen is located on this protein. An antibody with a specificity related to the Rh blood group system (R6A) showed $4{\cdot}6$–$10{\cdot}4 \times 10^4$ binding sites to be present on cells of phenotype cCDEe. On cells of phenotype -D- $1{\cdot}24 \times 10^4$ binding sites were present but protease treatment increased the number of available sites to $1{\cdot}3 \times 10^5$. An antibody to a Kell-related antigen (BRIC 18) recognized $2{\cdot}5$–$5{\cdot}9 \times 10^3$ sites per erythrocyte on cells of phenotype Kk. However, a similar number also appeared to be present on cells of the McLeod and Ko phenotypes although the affinity for the antigen on these cells was very much reduced. The potential of using monoclonal antibodies for this purpose and the value of this in the study of blood group systems has been demonstrated.

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INTRODUCTION

Recent developments in the production of monoclonal antibodies by hybridoma fusion techniques has led to the availability of a number of antibodies with specificities to antigens within several blood group systems (Fraser *et al.*, 1982; Anstee & Edwards, 1982; Parsons, Judson & Anstee, 1982; Ridgwell, Tanner & Anstee, 1983b). Such antibodies offer the possibility of extending our knowledge of blood group antigens in several ways. For example, immunoprecipitation studies of labelled membrane components from normal and abnormal cells have been used to help characterize antigens in several blood group systems. The function of the antigens can be examined by means of specific inhibition by monoclonal antibodies. Another area in which these antibodies are of potential value is in the quantification antigen sites on erythrocytes.

Numbers of antigen sites for the ABH (Economidou, Hughes-Jones & Gardner, 1967), I (Evans, Turner & Bingham, 1965), Lewis (Holburn, 1973), Kell (Hughes-Jones, Gardner & Lincoln, 1971) blood group systems have previously been estimated using radiolabelled polyclonal human antibodies and vary from 5×10^3 for Kell and Le^a to more than 10^6 for ABH antigens. The number of sialoglycoprotein α (SGP α, glycophorin A) molecules which carry the M and N antigens has been found to be 1×10^6 by a radioimmunoassay technique (Gahmberg, Jokinen & Andersson, 1979). Other studies have used ferritin-

labelled polyclonal antibodies to determine antigen site numbers for Rh, Duffy, Kidd, Diego and U blood group systems by means of electron microscopy (Masouredis *et al.*, 1980) and values from $1{\cdot}4 \times 10^4$ for Jk^a to $2{\cdot}3 \times 10^4$ for U were measured. In the latter case the complexity of the technique would considerably limit its general application. A major problem in the work with radiolabelled antisera has been the difficulty in purification of the specific antibody from the antiserum. This is performed by absorption of the antibody from the serum with erythrocytes positive only for the appropriate antigen followed by the elution of that antibody from the cell (Hughes-Jones *et al.*, 1971). This technique is difficult to perform and generally gives a low yield of purified antibody. In addition, there is always some non-specific binding of other antibodies to the cells used with resultant contamination of the desired antibody. Other problems may be encountered through the use of polyclonal antisera which may have a population of antibodies with related, but distinct, specificities to the same antigen. It has, for example, been reported by Chaplin, Monroe & Lachmann (1982) that in the case of two monoclonal antibodies with distinct specificities for C3 fragments, C3d and C3g, that each antibody can bind to C3 without steric hinderance to the other although the antigenic determinants are in close proximity on the polypeptide.

These problems could be minimised by the use of monoclonal antibodies where it is possible to obtain the antibody in a high degree of purity and where a single specificity for a binding site on the antigen will be present. The use of monoclonal antibodies to quantitate C3 bound to erythrocytes has previously been described (Merry *et al.*, 1984). In the present study the application of monoclonal antibodies to the quantitation of the number of antigen sites present on the erythrocyte surface for SGP α, the Wr^b antigen and Kell and Rhesus related antigens have been examined.

MATERIALS AND METHODS

Antibodies

Mouse ascites fluid containing antibody was prepared as previously described (Edwards, 1980; Parsons *et al.*, 1982). Briefly, BALB/c mice were given intraperitoneal injections of intact human erythrocytes and the spleen cells fused with NS1 cells. The resulting hybridomas were screened for the secretion of haemagglutinating antibodies and their specificity defined using a panel of rare erythrocytes expressing null phenotypes (Anstee & Edwards, 1982). Monoclonal antibodies LICR/LON R10, LICR/LON R18 and LICR/LON R6A were produced by Edwards (1980) and their specificity defined by Anstee & Edwards (1982). Monoclonal antibody NBTS/BRIC 18 was described by Parsons *et al.* (1982) and monoclonal antibodies NBTS/BRIC 13 and NBTS/BRIC 14 were described by Ridgwell *et al* (1983b). Monoclonal antibody NBTS/BRIC 8 was described by Holt *et al.* (in preparation) and Merry *et al.* (1984). Rabbit anti-mouse IgG was obtained from DAKO A/S Copenhagen (Code Z 109).

Labelling of antibodies

The ascites fluid was diluted to a concentration of 2·5–5 mg/ml protein with phosphate buffered saline pH 7·2 and iodinated by the chloramine-T method (Greenwood, Hunter & Glover, 1963) using ^{125}I (Code IMS 30) or ^{131}I from Amersham International. The anti-mouse IgG was similarly iodinated at a protein concentration of 10 μg/ml. Labelled ascites fluid was then passed through a $40 \times 1{\cdot}5$ cm column of Sephacryl S200 (Pharmacia Ltd.) to separate the immunoglobulin fraction. The protein concentration was monitored and antibody activity measured either by haemagglutination of appropriate cells or by incubation with the cells and estimation of bound radioactivity. The specific radioactivity was measured, assuming an absorbance of 1·43 for a 1 mg/ml solution of IgG, and the fractions containing antibody activity and having similar specific radioactivities pooled. The homogeneity of the recovered labelled IgG is illustrated for the monoclonal antibody R10 in Fig. 1 (a, b). Three antibodies of subclasses other than IgG1 (R18, BRIC 14 and BRIC 18) were purified on Protein A Sepharose (Pharmacia Ltd.). The antibodies were bound at pH 8·6 in 0·05 M Tris/HCl containing 0·15 M NaCl as described (Mishell & Shiigi, 1980) and eluted with 0·1 M citrate buffer pH 3·0. The purified IgG was analysed by SDS-PAGE (Fig. 1c–h). Purified antibodies ran as a major high molecular weight band which was shown to be IgG by immunoelectrophoresis. The IgG was then labelled with ^{125}I by the Chloramine T method and free and bound radioactivity separated by gel filtration on Sephadex G25.

Estimation of antigen site numbers

The labelled IgG fraction of the antibody was diluted in phosphate-buffered saline pH 7·2 in a medium

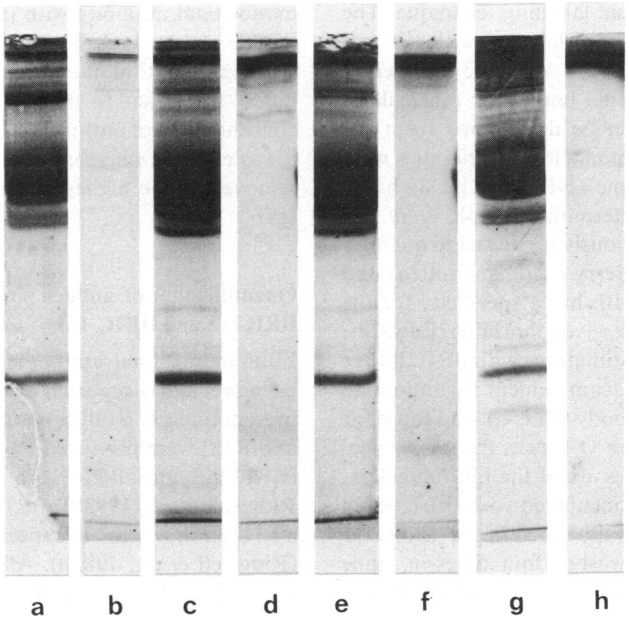

Figure 1. Analysis of purified antibody preparations by SDS polyacrylamide gel electrophoresis. Ascites fluid and purified antibodies were run without reduction on an 8% SDS-polyacrylamide slab gel with a 3% overlay using the buffer system of Laemmli (1970) and stained with Coomassie Blue.

(a) R10 ascites fluid (2·5 μl); (b) R10 purified by gel filtration on Sephacryl S200 (5 μg); (c) R18 ascites fluid (2·5 μl); (d) R18 affinity purified on Protein A Sepharose (25 μg); (e) BRIC 14 ascites fluid (2·5 μl); (f) BRIC 14 affinity purified (63 μg); (g) BRIC 18 ascitic fluid (2·5 μl); (h) BRIC 18 affinity purified (25 μg).

containing 2·25% human albumin. The concentration in each dilution was estimated from the specific radioactivity. Duplicate samples of 0·1 ml from each dilution were then incubated with 0·1 ml of a 10% suspension of the cells in phosphate-buffered saline pH 7·2 for 2 hr at 37°. In incubations with antibodies to SGP α a 5% cell suspension was used and the tubes were agitated frequently to break up the agglutinates. In these experiments siliconised tubes were also used as the agglutinated cells tended to stick to the tubes. They were then washed five times in saline, transferred to new tubes and the radioactivity counted. An accurate cell count was then performed on a Coulter Counter. The maximum number of antibody molecules bound was determined from a Scatchard Plot (Scatchard, 1949) and from the cell count the number of antibody molecules per cell calculated. A 1:1 binding ratio of monoclonal antibody to antigen was used to give the number of antigen sites.

Erythrocytes

En(a-) erythrocytes (donor M.E-P) were kindly provided by Dr W. Wagstaff, Regional Transfusion Centre, Sheffield. Ko erythrocytes (E.B.) and McCleod erythrocytes (donors R.F. and R.E.) by Mrs M. Leak, South London Transfusion Centre, Tooting. Rh null erythrocytes (Y.T.) by Dr P. Harden, Red Cross Blood Transfusion Service, Queensland, Brisbane, Australia. -D- (G.L.), ·D· (N.E.), (H.D.) erythrocytes by Dr M. Contreras, North London Transfusion Centre, Edgware, Middlesex.

Treatment with 2-aminoethylisothiouronium bromide was carried out as described by Advani *et al.* (1982), and treatment with pronase by incubation with 1 mg/ml enzyme (Koch-Light) in 0·15 M NaCl containing 1 mm $CaCl_2$ for 30 min at 37°.

RESULTS

Binding of anti-mouse IgG to monoclonal antibodies

To use a second antibody technique for the estimation of number of antigenic sites it is first necessary to find the ratio of binding between the two antibodies. This

may be measured by a dual labelling technique. The anti-mouse IgG antibody was labelled with ^{125}I and the monoclonal antibody with ^{131}I. The number of molecules of anti-mouse IgG bound per molecule of monoclonal IgG can then be determined from the ratio of ^{125}I to ^{131}I. Two monoclonal antibodies were chosen for this study. One (NBTS/BRIC 8) has a specificity for a C3d determinant (Holt *et al.*, in preparation) and has previously been used to quantify erythrocyte-bound C3 (Merry *et al.*, submitted) and the other (LICR/LON R10) has a specificity for the sialoglycoprotein α (SGP α, glycophorin A) (Edwards, 1980). The cells used for estimations with BRIC 8 were previously sensitized with complement by an human anti-blood group Le^a antibody (ELe^aC), and those for estimations with R10 were O+ cells from a normal individual. Both antibodies are of the IgG1 subclass.

Erythrocytes were first incubated with ^{131}I-labelled mouse monoclonal IgG as described in the 'Methods' section. They were then washed four times in saline and incubated with ^{125}I-labelled anti-mouse IgG at concentrations of 10·9 to 1400 μg/ml and washed a further four times. The amount of monoclonal antibody bound was then calculated from the ^{131}I radioactivity and that of anti-mouse IgG from the ^{125}I radioactivity. In this way any elution of the monoclonal antibody from the cell may be measured. Table 1 shows the ratio of anti-mouse IgG bound to monoclonal antibody with increasing concentrations of anti-mouse IgG. It is apparent that the ratio of binding of anti-mouse IgG to the BRIC 8 antibody differs from that to the R10 antibody at any given concentration of anti-mouse IgG. No elution of BRIC 8 was evident, whereas 75% of the R10 antibody was removed at the highest concentration of anti-mouse IgG.

Quantification of antigen sites on SGP α (R10, R18, BRIC 13 and BRIC 14)

Four monoclonal antibodies were used which have been previously reported as recognizing determinants present on SGP α (glycophorin A). Two of these (R10 and R18) were produced by Edwards (1980) and two (BRIC 13 and BRIC 14) previously described by Ridgwell *et al.* (1983b). BRIC 13 and BRIC 14 have also been shown to have specificity for the Wr^b antigen (Ridgwell *et al.*, 1983b). All these antibodies agglutinate normal cells but not En(a-) cells which lack SGP α. R10, R18 and BRIC 14 also precipitate SGP α from normal erythrocytes. The antibodies were directly labelled with ^{125}I and the immunoglobulin fraction isolated as described in the 'Methods' section. The specific radioactivity was then determined and the maximum number of binding sites calculated from a Scatchard Plot. The values obtained for normal

Table 1. Binding ratio of anti-mouse IgG to monoclonal antibodies BRIC 8 and R10

	μg anti-mouse IgG added	μg anti-mouse IgG bound	μg mouse IgG bound	Ratio anti-mouse /mouse IgG
(a) R10	21·4	0·370	0·321	1·15
	43·75	0·616	0·328	1·88
	87·5	0·920	0·331	2·78
	175	1·190	0·322	3·70
	350	1·164	0·260	4·48
	700	0·958	0·189	5·06
	1400	0·358	0·080	4·45
(b) BRIC 8	21·4	0·175	0·380	0·46
	43·75	0·352	0·393	0·90
	87·5	0·666	0·422	1·58
	175	1·254	0·474	2·65
	350	1·545	0·432	3·57
	700	1·943	0·456	4·26
	1400	2·515	0·422	5·96

Either unsensitized cells (R10) or C3 sensitized cells (BRIC 8) were incubated with the monoclonal antibody, washed and then incubated with the anti-mouse IgG. Anti-mouse IgG was labelled with ^{125}I and the mouse IgG with ^{131}I as described in the Methods section. Counts were measured simultaneously on dual-channel gamma counter.

erythrocytes and for En(a-) erythrocytes are shown in Table 2. The low number of binding sites present on the EN(a-) cells confirms the specificity of these antisera and although there is considerable variation in the absolute number of sites recognized by each monoclonal antibody the values are in the same order of $0{\cdot}3–1{\cdot}2 \times 10^6$ sites per cell. Values for the equilibrium constant for each antibody were also calculated (Table 2). The number of SGP α sites measured using antibodies R18 and BRIC 14 purified by affinity chromatography on Protein A Sepharose were not substantially different from those obtained with antibodies purified by gel filtration (Table 2).

Table 2. Number of antigen sites for antibodies to sialoglycoprotein

Antibody	Sites per erythrocyte ($\times 10^5$) Normal	En(a-)	K_{Eq} (M^{-1}) ($\times 10^6$)
R10	4·7–6·9 (10)	0·11	1·4
R18	5·7–7·8 (3)	0·06	0·5
BRIC 13	9·9–11·9 (3)	0·10	0·8
BRIC 14	7·9–10·6 (3)	0·05	0·4
R18 Affinity-purified	4·2–6·9 (6)	0·01	3·4
BRIC 14 Affinity-purified	3·2–5·2 (7)	0·05	0·4

Values in parentheses represent the number of estimations performed. The number of antigen sites and the equilibrium constants were calculated from Scatchard plots. The En(a-) cells were from donor M.E-P.

Quantification of sites for monoclonal antibody R6A

A monoclonal antibody (R6A) has been produced by Edwards (1980) which has a specificity apparently related to the Rh system as it fails to agglutinate Rh_{NULL} cells (Anstee & Edwards, 1982). The number of antigen sites recognised by this antibody were quantified as described in the 'Methods' section and the maximum amount bound was calculated from a Scatchard Plot. Table 3 shows the number of sites recognized by this antibody on various cells including -D-, ·D· and Rh_{NULL} and also shows the effect of prior protease treatment of the cells on the number of sites determined. The number of sites determined on normal cells (phenotype cCDEe) was in the range of $4{\cdot}6–10{\cdot}4 \times 10^4$. The specificity of the antibody is shown by the low number of sites present on Rh_{NULL} cells. The effect of prior protease treatment on the number of sites for R6A available on homozygous -D- cells was striking in that it revealed binding sites for the R6A not present on unmodified cells (Table 3).

Table 3. Number of antigen sites for R6A

Rhesus phenotype of cells	Sites per erythrocyte $\times 10^{-4}$ Untreated	Pronase-treated
cCDEe	4·6–10·4	7·1
Rh_{NULL}	0·69	0·85
-D-	1·24	13·3
·D·	0·96	n.d.

The number of antigen sites were calculated from Scatchard plots. The values for cells of presumptive genotype R_1R_2 are the range for 4 examples tested. Values for Rh_{NULL} (Y.T.), -D- (G.L.) and ·D· (N.E.) erythrocytes are single estimations.

Quantification of sites for monoclonal antibody BRIC 18

The number of sites recognised by the monoclonal antibody BRIC 18 was determined. This antibody has an apparent specificity for a Kell-related antigen since it will not agglutinate Ko cells which are thought to lack all antigens of the Kell blood group system and only reacts weakly with cells of McLeod phenotype which have only very weak expression of Kell system antigens (Parsons *et al.*, 1982). The number of binding sites detected on normal cells (phenotype Kk) is shown in Table 4. It also appears, however, that a similar number of binding sites are present on cells of Ko and McLeod phenotype though there is a great difference in the equilibrium constant calculated from the Scatchard plots shown in Fig. 2. The binding to cells treated with AET which is reported to inactivate specifically all antigens of the Kell blood group system (Advani *et al.*, 1982) was not greatly reduced (Table 4) although the equilibrium constant was markedly lower than that for normal cells (Fig. 2). Values for the number of antigen sites determined using the affinity-purified antibody on normal and on AET-treated cells were slightly less than for antibody purified by gel filtration (Table 4). The value obtained for the equilibrium constant for the binding of BRIC 18 to normal cells was greater when labelled affinity-purified antibody was used (Table 4).

Table 4. Number of antigen sites for BRIC 18

Cell type	Sites per erythrocyte $\times 10^{-3}$	K_{Eq} (M^{-1})
Normal (phenotype Kk)	3·1–5·9 (2·5)	$8{\cdot}6 \times 10^6$ (5×10^7)
McLeod	4·2, 4·5	$0{\cdot}5 \times 10^6$
Ko	3·9	$0{\cdot}3 \times 10^6$
Normal AET-Treated	2·9 (1·1)	$1{\cdot}2 \times 10^6$ (1×10^6)

Values for the number of antigen sites and for the equilibrium constants were calculated from the Scatchard plots shown in Fig. 2, except for the numbers in parentheses which were obtained using affinity purified BRIC 18. Values for site numbers on normal cells represent the range for single determinations on four different cells, those given for McLeod cells are single determinations on donors R.E. and R.F. respectively.

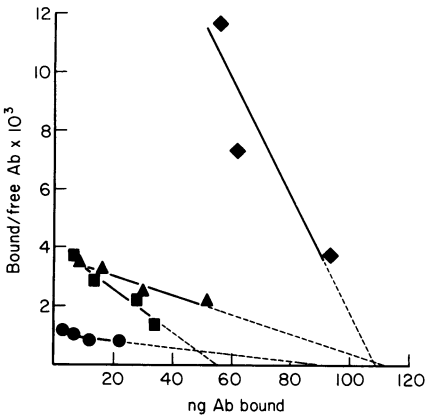

Figure 2. Scatchard Plots for binding of BRIC 18 to normal, McLeod, Ko and AET-treated cells. Cells were incubated with different concentrations of ^{125}I-labelled BRIC 18 as described in the 'Methods' section and the amount bound to the cells calculated. (♦) Normal; (▲) McLeod; (●) Ko; (■) AET-treated.

DISCUSSION

The most simple and convenient way to estimate the number of antigen sites recognized by monoclonal mouse antibodies would be to use a labelled anti-mouse IgG antibody in a second antibody technique. Unfortunately, the results obtained with two monoclonal antibodies shown in Table 1 demonstrate that this is not possible. With the anti-C3d BRIC 8, very high concentrations of anti-mouse IgG had to be used before the point at which the binding to the mouse IgG became saturating and it would, therefore, be impossible to apply a single binding ratio to calculate the amount of bound mouse antibody. With the anti-SGP α it was found that the ratio reached a maximum value but the amount of mouse IgG remaining bound after addition of the anti-mouse IgG was reduced by 75% at the highest concentration of the anti-mouse IgG. This suggests that the complex of the two antibodies is eluting from the cells and this is unsuitable for accurate quantification measurements. It is possible that a similar effect is responsible for the prozone phenomenon observed with anti-human IgG antisera when titrated against cells weakly sensitized with IgG. In view of these results the only approach to the problem is to directly label the monoclonal antibody.

The results obtained with the antibodies R10, R18, BRIC 13 and BRIC 14 shown in Table 2 are of a similar order of magnitude. The relatively wide range in the number of sites detected may partly reflect the error in estimation. Several difficulties were encountered which are probably associated with the large number of sites present. The main problem was intense agglutination of the cells by some of the antibodies. In addition, the method of calculation using the Scatchard plot becomes less accurate at such large site numbers as the degree of extrapolation is greater. The results obtained showing a range of $0{\cdot}3$–$1{\cdot}2 \times 10^6$ sites per cell is, however, in the same order as that previously found for the number of SGP α sites using a competitive binding radioimmunoassay technique with isolated protein (Gahmberg *et al.*, 1979). R10 and R18 are known to react with SGP α. The antibodies BRIC 13 and BRIC 14 have anti-Wr^b specificity. The observation that the number of binding sites for these antibodies is of the same order as that for R10 and R18

is consistent with previous observations (Ridgwell *et al.*, 1983b) that localized this antigen to SGP α.

Studies with the monoclonal antibody R6A showed that it was detecting a determinant not present on Rh Null cells (Table 3), confirming its specificity for a Rhesus-related antigen. As yet there is little known of the nature of this antigen although immune precipitation (using R6A) of membrane components from cells radioiodinated with lactoperoxidase had identified an R6A binding polypeptide of apparent molecular weight 34,000. Under comparable conditions human anti-D bound to a component of molecular weight 32,000 (Ridgwell *et al.*, 1983a) and this component corresponds to the Rh(D) polypeptide identified previously by Moore *et al.* (1982) and Gahmberg (1982). Both the 34,000 and the 32,000 molecular weight components contain extracellular sulphydryl groups which can be labelled with the impermeant malemide N-maleoylmethionine (35-S) sulphone in normal cells but not in Rh null cells. The number of R6A binding sites detected here on cells of rhesus phenotype CcDEe ($4{\cdot}6$–$10{\cdot}4 \times 10^4$, Table 3) is of the same order as that found for the c̄ antigen on homozygous cc cells ($7{\cdot}5$–$8{\cdot}2 \times 10^4$) by Hughes-Jones & Gardner (1971) and for the C antigen on homozygous CC cells ($4{\cdot}2$–$5{\cdot}6 \times 10^4$) by Skov & Hughes-Jones (1977) using polyclonal antibodies and higher than that obtained for the D and E antigens ($1{\cdot}5$–$3{\cdot}5 \times 10^4$) Hughes-Jones & Gardner (1971). These results support the conclusion that R6A binds to a different polypeptide from that which carries the D antigen and suggests the possibility that the c̄C antigens are located on the R6A binding polypeptide. The conclusion that R6A reacts with a determinant distinct from the Rh(D) polypeptide is further supported by the results obtained with homozygous -D- and homozygous ·D· cells, both of which have an elevated number of D antigen sites ($1{\cdot}1$–$2{\cdot}02 \times 10^5$) (Hughes-Jones *et al.*, 1971) but have a markedly reduced number of R6A binding sites. Previous studies have indicated that protease treatment of homozygous -D- cells restores their agglutinability with R6A to normal levels (Anstee & Edwards, 1982). The results of quantitative analysis presented here demonstrated an increase in the number of sites detected on -D-/-D- cells from $1{\cdot}24 \times 10^4$ to $1{\cdot}33 \times 10^5$ after pronase treatment of the intact cells. This suggests that the R6A epitope is a cryptantigen on the untreated homozygous -D- cells and accessibility to R6A is hindered by the increased number of Rh(D) antigen sites present.

Reactions of the BRIC 18 antibody (Table 4) are difficult to interpret in view of the binding to Ko and AET-treated cells in which all antigens of the Kell system are believed to be absent and inactivated respectively and also because of the small number of molecules bound. However, the number of BRIC 18 binding sites obtained for normal erythrocytes ($2{\cdot}5$–$5{\cdot}9 \times 10^3$) is in excellent agreement with values published for human anti-K (Hughes-Jones & Gardner, 1971). The marked reduction in affinity of BRIC 18 for Ko, McLeod and AET-treated cell raises the intriguing possibility that the BRIC 18 binding component is not absent in these cells but is present in an abnormal form for which the antibody has only a low affinity. Thus, it may not agglutinate the cells but still binds to the antigen and is therefore detected when radiolabelled antibody is used. Such an altered form would be inherited in the case of cells of the Ko and McLeod phenotypes but would have to result from the chemical modification of the protein in the case of AET-treated cells.

The results presented here clearly show that monoclonal antibodies may be used to quantify erythrocyte antigens but only by a direct technique with labelled antibody. As more monoclonal antibodies become available valuable information will be gained from quantitation of the abundance of antigens present on normal and abnormal cells. Such information, together with the identification and characterisation of the blood group active components involved, should add substantially to our understanding of the topography of the red cell surface and the complexity of blood group systems.

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