Carbohydrate Research, 178 (1988) 203–212
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

EFFECT OF ENDOGLYCOSIDASE F–PEPTIDYL *N*-GLYCOSIDASE F PREPARATIONS ON THE SURFACE COMPONENTS OF THE HUMAN ERYTHROCYTE*

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(Received July 1st, 1986; accepted for publication in revised form, May 22nd, 1987)

ABSTRACT

Endo-*N*-acetyl-β-D-glucosaminidase F–Peptidyl *N*-glycosidase F preparations (abbreviated Endo F) and endo-β-D-galactosidase were used to study the major human erythrocyte membrane glycoproteins and the components carrying the blood group A, B, Rhesus (D), and Duffy (Fy^a) antigens. The results are consistent with the known presence of an *N*-glycosyl-linked oligosaccharide on sialoglycoprotein α and the absence of such an oligosaccharide from sialoglycoprotein δ. Under the conditions used, only a portion of the *N*-glycosyl-linked oligosaccharides on band 3 molecules were cleaved by Endo F alone or by Endo F in combination with endo-β-D-galactosidase. Immunoblotting experiments showed that treatment of red cells with Endo F alone had little effect on the components carrying blood group A and B antigen activity. However, Endo F used in combination with endo-β-D-galactosidase caused a substantial reduction in the binding of monoclonal anti-A and anti-B antibodies. The results clearly show that sialoglycoproteins α and δ carry little or no blood group A or B activity. Endo F alone, or in combination with endo-β-D-galactosidase, had no effect on the electrophoretic mobility of the Rh(D) polypeptide, supporting previous suggestions that this membrane polypeptide is unusual in not being glycosylated. Endo F had a dramatic effect on the electrophoretic mobility of the component(s) carrying blood group Fy^a activity. The diffuse Fy^a component of M_r 38 500–90 000 was sharpened to a band of M_r 26 000. Either endo-β-D-galactosidase or neuraminidase treatment reduced the M_r of the Fy^a component(s) but did not significantly sharpen the bands, suggesting that the Fy^a component contains between 40–50% by mass of *N*-glycosyl-linked oligosaccharides.

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INTRODUCTION

Elder and Alexander[1] have shown that cultures of *Flavobacterium meningosepticum* produce an endoglycosidase activity with broad specificity towards *N*-glycosyl-linked oligosaccharides. Plummer *et al.*[2] subsequently showed that these preparations also contain a peptidyl-*N*-glycosidase F. It was of interest to study the effect of this preparation containing both enzymes (abbreviated Endo F) on the red cell-surface glycoproteins of the human erythrocyte and the blood group antigens associated with them. We have reported, elsehwere, the effect of these enzymes on the blood group antigens LW (ref. 3), and Lutheran (Lu^b) (ref. 4). In this paper, we describe the effects of Endo F preparations on the major human erythrocyte-membrane glycoproteins and on those membrane components which carry the blood group A, B, Rh(D), and Duffy (Fy^a) antigens.

EXPERIMENTAL

Materials and methods. — The surface of human erythrocytes was labelled with the periodate–NaB^3H_4 method, as described[5], and the lactoperoxidase radioiodination method[6]. $NaDodSO_4$–polyacrylamide gel electrophoresis of erythrocyte membranes was carried out, on gels containing 10% acrylamide, by the method of Laemmli[7]. The method used for immunoblotting with murine monoclonal antibodies was as described[3]. When human antibodies were used, the binding was detected with biotinylated Protein A (1 μL/cm^2 of nitrocellulose) and streptavidin–horse-radish peroxidase conjugate as described by the manufacturer (Amersham International PLC, Bucks, U.K.)

Murine monoclonal antibody LICR/LON R1.3 (R1.3) has been described previously[8]. The murine hybridoma line, secreting the monoclonal anti-blood group B antibody NBTS/BRIC 30 (BRIC 30), was cloned from a fusion performed by use of spleen cells from a mouse given twelve intraperitoneal injections of human group B erythrocytes. Each injection comprised 100 μL of a 50% suspension of erythrocytes in PBS, pH 7.3, given once weekly for twelve weeks. Fusion was performed three days after the twelfth injection. BRIC 30 (IgM class) was not inhibited by either 0.1M D-galactose or 0.1M *N*-acetyl-D-galactosamine, but was absorbed by Synsorb B (Chembiomed Corp., Alberta, Canada). Murine monoclonal anti-blood group A antibody (ES-9) was a gift from Dr. S. Moore, South East Scotland Regional Transfusion Service, Edinburgh. ES-9 is of the IgM class and is absorbed by Synsorb A (ref. 9); it was not inhibited by 0.1M *N*-acetyl-D-galactosamine. Synsorb A and Synsorb B have either blood-group A or blood-group B active structures attached to an insoluble support. The structures are α-D-Gal*p*NAc-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-Sup and α-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→2)-β-D-Gal*p*-Sup, respectively, where Sup is an insoluble silica carrier component. Human anti-D (Dod) serum was a gift from Dr. C. Hodson, Lancaster Blood Transfusion Service, Lancaster. Human anti-Fy^a serum (Gon) was available at the South

Western Regional Transfusion Centre, Bristol. Anti-Fy^a was prepared for immunoblotting by a method modified from that of Kochwa and Rosenfield[10]. Human Fy(a+) erythrocytes (1 vol.) were incubated with human anti-Fy^a serum (4 vol.), either overnight at 4° or for 1.5 h at 37°. The erythrocytes were washed with 0.15M NaCl, made to 10% in ice-cold 0.15M NaCl, and ice-cold 1% digitonin (0.5 vol.; Sigma Chemical Co. Ltd., Poole, Dorset) in 0.15M NaCl was added. The resulting precipitate was collected by centrifugation at 9000 *g* for 2–5 min, the supernatant discarded, 0.1M glycine·HCl–0.15M NaCl (pH 2.5; 2 vol.) added to the precipitate, and the whole incubated for 5 min on ice prior to a further centrifugation at 9000*g* for 2–5 min. The resulting supernatant containing anti-Fy^a antibody was collected, the pH adjusted to 7.0 with 0.5M Tris, and the mixture used for immunoblotting.

Endo-β-D-galactosidase was prepared and used as described by Mallinson *et al.*[3]. The enzyme was dialysed against 2mM sodium acetate, pH 6.0, containing 2mM calcium acetate and lyophilised. Routinely, the packed red cells (100 μL) were incubated with this preparation (100 μg dry-weight) at 37° overnight. Endo F preparations were obtained as described by Elder and Alexander[1] from cultures of *F. meningosepticum* as modified below. The culture supernatant was saturated at 80% with ammonium sulphate, and the precipitate collected by centrifugation at 30 000 *g* for 30 min. The precipitate was dissolved in 500mM EDTA, pH 7.4, and dialysed against 5mM EDTA, pH 7.4, overnight at 4°. The material was re-precipitated with ammonium sulphate and the fraction precipitating between 30% and 80% of saturation with ammonium sulphate was collected by centrifugation at 30 000 *g* for 30 min. This material was dissolved in 50mM EDTA (pH 8.0, 1.5 mL) and clarified by centrifugation at 30 000 *g* for 10 min before being applied to a column of Sephacryl S200 (89 cm × 2.2 cm), equilibrated with 5mM EDTA, pH 8.0. Endo F activity was detected by incubating each column fraction (1 vol.) for 18 h at 37° with a solution containing 0.2M Na_3PO_4, 100mM EDTA, 1% Nonidet P40, 2% 2-mercaptoethanol ovotransferrin (0.25 mg/mL), and ovalbumin (1 mg/mL, 2 vols.; final pH 6.1). The incubated samples were separated by gel electrophoresis on gels containing $NaDodSO_4$ and 8% acrylamide. Endo F activity was detected by the shift in M_r of the ovotransferrin and ovalbumin bands after deglycosylation. Fractions which showed Endo F activity and no net loss due to proteolysis of the ovotransferrin and ovalbumin bands were pooled and concentrated by pressure dialysis through an Amicon PM-10 filter. Endo F digestion of erythrocytes was as described by Mallinson *et al.*[3]. Routinely, packed red cells (500 μL) were incubated with Endo F (10–20 μL depending on the preparation) overnight at 37°. Immunoprecipitation of the Rh(D) polypeptide was carried out as described by Moore *et al.*[6]. Endo F digestion of the solubilised Rh(D) polypeptide were carried out as follows: The immunoprecipitate was solubilised in 5% $NaDodSO_4$ and 1 vol. mixed with 19 vols. of a solution containing 0.2M NaH_2PO_4, 100mM EDTA, and 1% Nonidet P40 (pH 6.0), and 2-mercaptoethanol was added to a concentration of 2% and phenylmethanesulphonyl fluoride to a concentration

of 1mM. Endo F preparation (1 μL) was added and incubated for 6.5 h at 37°. As a control, ovalbumin (0.25 mg/mL) and ovotransferrin (0.5 mg/mL) were Endo F-treated under the same conditions (final $NaDodSO_4$ concentration 0.25%) and showed the expected shifts in M_r on $NaDodSO_4$–polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Effects on major red-cell-membrane sialoglycoproteins. — Red cell-surface glycoproteins were labelled by the periodate–NaB^3H_4 method (which selectively labels the red cell sialoglycoproteins) and by the lactoperoxidase-catalysed radioiodination (which labels bands 3, 4, 5, and sialoglycoproteins α and δ, but not sialoglycoproteins β and γ; see Anstee *et al.*[11] for nomenclature). Periodate–NaB^3H_4-labelled red blood cells were treated with Endo F, endo-β-galactosidase, and a mixture of both enzymes (Fig. 1a–e). Endo-β-galactosidase had no effect on the mobility of any of the sialoglycoproteins. Endo F (with or without endo-β-galactosidase) caused a marked reduction in M_r of the monomer of sialoglycoprotein α (from 43 000 to 40 000) and the complexes involving sialoglycoprotein α (α_2, from 86 000 to 81 000; and $\alpha\delta$, from 68 000 to 66 000). No change in mobility

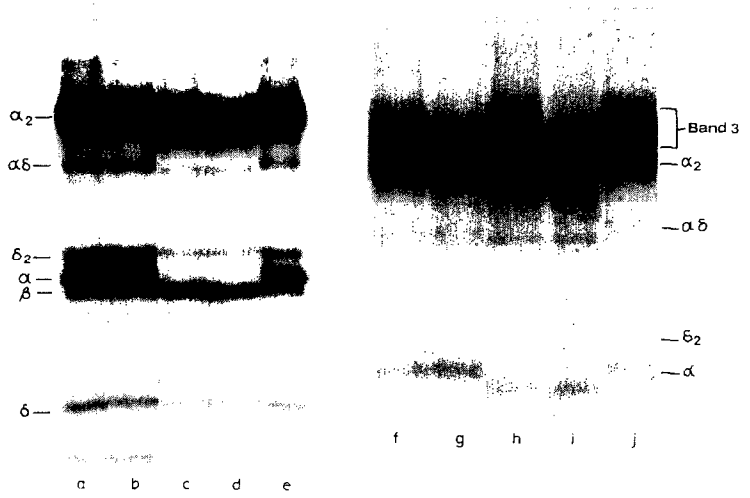

Fig. 1. Effect of endoglycosidases on the mobility of surface-labelled proteins of human red cells. Tracks (a–e). Effects on red cells labelled by the periodate–NaB^3H_4 method: (a) and (e) Normal erythrocytes incubated in the absence of glycosidase; (b) erythrocytes incubated with endo-β-galactosidase; (c) erythrocytes incubated with Endo F; and (d) erythrocytes incubated with endo-β-galactosidase plus Endo F. Tracks (f–j). Effects on red cells labelled by lactoperoxidase-catalysed radioiodination: (f) and (j) Normal erythrocytes incubated in the absence of glycosidase; (g) erythrocytes incubated with endo-β-galactosidase; (h) erythrocytes incubated with Endo F; and (i) erythrocytes incubated with endo-β-galactosidase plus Endo F.

was observed for the bands corresponding to sialoglycoproteins δ or δ_2. In addition, extended exposure of the fluorographs did not show any change in the mobility of sialoglycoprotein γ or the broad band containing sialoglycoprotein β_1 (data not shown). Since Endo F-treated sialoglycoprotein α comigrated with sialoglycoprotein β, it was not possible to assess the degree of degradation of intact sialoglycoprotein β. In the experiment described herein, a very faint band was observed in the region corresponding to Endo F-degraded sialoglycoprotein β. When the cells were labelled by lactoperoxidase-catalysed radioiodination, the effect of Endo F on the α, $\alpha\delta$, and α_2 bands was clearly apparent with no effect on the δ and δ_2 bands (Fig. 1f–j).

The monoclonal antibody R1.3 reacts with the *N*-terminal portion of both sialoglycoproteins[8,13] α and δ. Immunoblotting using R1.3 on membranes from endo-β-galactosidase-treated erythrocytes showed no change in the mobility of any of the sialoglycoproteins α- or δ-containing bands as expected (Fig. 2). However, membranes from cells treated with a mixture of Endo F and endo-β-galactosidase showed a marked shift in the sialoglycoprotein α-containing bands (α, $\alpha\delta$, and α_2), but no change in δ and δ_2 bands. The just mentioned results are consistent with the presence[14] of an *N*-linked oligosaccharide in sialoglycoprotein α, and the absence[15] of this type of oligosaccharide in sialoglycoprotein δ.

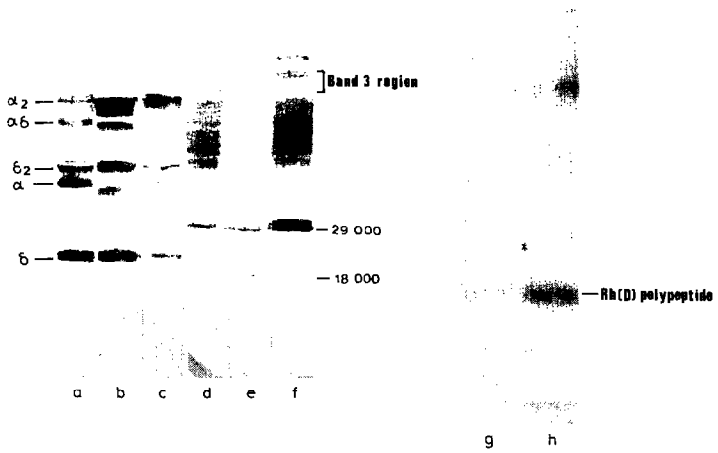

Fig. 2. Effect of endoglycosidases on human red cell-membrane sialoglycoproteins and blood group B- and Rh(D)-active components. Tracks (a–c). Immunoblotting with monoclonal antibody R1.3: (a) Membranes from endo-β-galactosidase-treated cells; (b) membranes from cells treated with endo-β-galactosidase plus Endo F; and (c) membranes from cells incubated in the absence of glycosidase. Tracks (d–f). Immunoblotting with monoclonal anti-blood group B antibody (BRIC 30): (d) Membranes from cells treated with endo-β-galactosidase; (e) membranes from cells treated with endo-β-galactosidase plus Endo F; and (f) membranes from cells incubated in the absence of glycosidase. Tracks (g,h). Immunoprecipitation of Endo F-treated, radioiodinated erythrocytes with human anti-D serum: (g) Immunoprecipitate from cells incubated in the absence of glycosidase; and (h) immunoprecipitate from cells treated with Endo F plus endo-β-galactosidase. The bands marked with an asterisk in track (h) resulted from contamination from a strongly-labelled sample in the adjacent track.

Sialoglycoprotein β contains an *N*-glycosyl-linked oligosaccharide[16], and we have previously shown by immunoblotting with a monoclonal anti-β antibody that it is relatively resistant to Endo F treatment of red cells[17]. In the experiments described herein, the extent of digestion could not be assessed because sialoglycoprotein β comigrates with the Endo F-digested sialoglycoprotein α. The absence of any observed effect of the enzyme preparation on β_1 or γ bands may result either from a similar resistance to degradation of an *N*-glycosyl-linked oligosaccharide or the lack of such an oligosaccharide.

Effects on other surface membrane components. — Lactoperoxidase-radioiodinated red blood cells were used to examine the effects of the endo-glycosidases on the major surface components. The anion-transport protein (band 3) was sharpened by treatment of red cells with endo-β-galactosidase alone, but there was only a slight decrease in the M_r of the leading edge of the bands, as was first shown by Mueller *et al.*[18]. Band 3 remained broad when the cells were treated with Endo F alone, although the leading edge of the band was shifted from an M_r of 92 000 to an M_r of 88 000. It appears that a proportion of the largest-band 3 oligosaccharide chains are relatively resistant to cleavage by Endo F alone. However, the combination of Endo F and endo-β-galactosidase sharpened the band more than endo-β-galactosidase or Endo F alone, and also shifted the front edge of the band to an M_r of 88 000. Band 3 remained relatively broad even on treatment with a combination of endo-β-galactosidase and Endo F, suggesting that deglycosylation was incomplete under these conditions. However, it was not possible to reduce the width of the band further, even under the most severe conditions of digestion we were able to achieve.

Extended autoradiography of the gels showed that the diffuse radioiodinated band in the "4.5" region was noticeably sharpened by endo-β-galactosidase treatment and was both sharpened and reduced in M_r by the combination of endo-β-galactosidase and Endo F (data not shown). This is consistent with the results of Mueller *et al.*[18] and the effects of these enzymes on the purified glucose transporter[19] which is a major component of the band 4.5 region.

Effects on A, B, Rh(D), and Duffy components. — Immunoblotting experiments used a mouse monoclonal anti-blood group B antibody (BRIC 30). Untreated erythrocytes of blood group B contained a broad spectrum of B antigen-containing components (Fig. 2). A large proportion of the binding was to a diffuse area covering M_r 45 000–85 000, which included the band 4.5 region. In addition, the higher M_r portion of band 3 bound the antibody. A major staining component on the gels had M_r 29 000, and there was a further component of M_r 18 000 which bound the antibody. There was no binding of anti-B antibody to sialoglycoprotein δ. Weak binding of the antibody was observed to a band having a mobility similar to that of α band, but this represented a very small proportion of the total binding of the antibody.

Endo F treatment of the cells did not markedly change the overall pattern of binding of anti-B antibody. In contrast, endo-β-galactosidase alone caused a notice-

able reduction of binding to all the bands, which was especially marked in the case of band 3. The combination of endo-β-galactosidase and Endo F resulted in the loss of binding to all the components. The reduction in binding of the 29 000 and 18 000-dalton bands was apparent in some experiments, but not in others. Comparable results (not shown) were obtained with a mouse monoclonal antibody against the blood group A (ES-9) antigen by use of membranes from blood group A erythrocytes.

Previous studies of Järnefelt *et al.*[20] and Finne[21] have indicated that ABH activity is carried on band 3 and band 4.5 in the erythrocyte membrane. Using a lectin (BS-I from *Bandeirea simplificolia*) which binds to blood group A, B, and AB erythrocytes, Finne[21] also showed binding to bands α and α_2, but not to band δ, and he suggested that band α carried A and B blood group antigenic activity. However, the anti-H lectin from *Lotus tetragonolobus* did not bind to bands α and α_2 in blood group O erythrocytes. BS-I lectin has a specificity for terminal 2-acetamido-2-deoxy-α-D-galactopyranosyl and α-D-galactopyranosyl groups[22], whereas the monoclonal antibodies used in the present study recognise structures additional to the terminal, nonreducing monosaccharide groups in the A and B determinants (see Experimental section). Our results with these more specific reagents suggest that sialoglycoprotein α contains little, if any, of the ABH activity of the erythrocyte. Takasaki and Kobata[23] reported that *N*-acetylgalactosamine could be incorporated into the *O*-glycosyl-linked oligosaccharides of preparations containing sialoglycoprotein α by use of a source of *N*-acetylgalactosaminyltransferase. These authors interpreted this result to imply that ABH-active determinants were naturally present in these oligosaccharides in the sialoglycoprotein. Our results using monoclonal antibodies clearly show that little, or no, ABH activity is found on the *O*-linked oligosaccharides of sialoglycoproteins α and δ, since there was no binding in the region of Endo F-treated band α in the membranes from cells that had been treated with both Endo F and endo-β-galactosidase (Fig. 2).

Gahmberg[24] suggested that the Rh(D) polypeptide is not glycosylated since it did not bind to various lectin–Sepharose columns, and its electrophoretic mobility was unaffected by treatment with either Endo H or endo-β-galactosidase. Treatment of intact radioiodinated erythrocytes with Endo F alone (not shown) or with Endo F in combination with endo-β-galactosidase (Fig. 2), followed by immunoprecipitation using human anti-D, did not result in any change in the M_r of the Rh(D) polypeptide. In order to establish that this resistance to Endo F was not due to steric hindrance at the cell surface, immunoprecipitates from radioiodinated, untreated intact erythrocytes were solubilised in sodium dodecyl sulfate and 2-mercaptoethanol, and incubated with Endo F. Incubation under these denaturing conditions did not alter the M_r of the Rh(D) polypeptide, thus confirming that the polypeptide does not carry any *N*-glycosyl-linked oligosaccharides (data not shown).

The Duffy blood group antigens (Fy^a and Fy^b) are known to be located on diffusely migrating membrane components having a M_r ~40 000, and are of par-

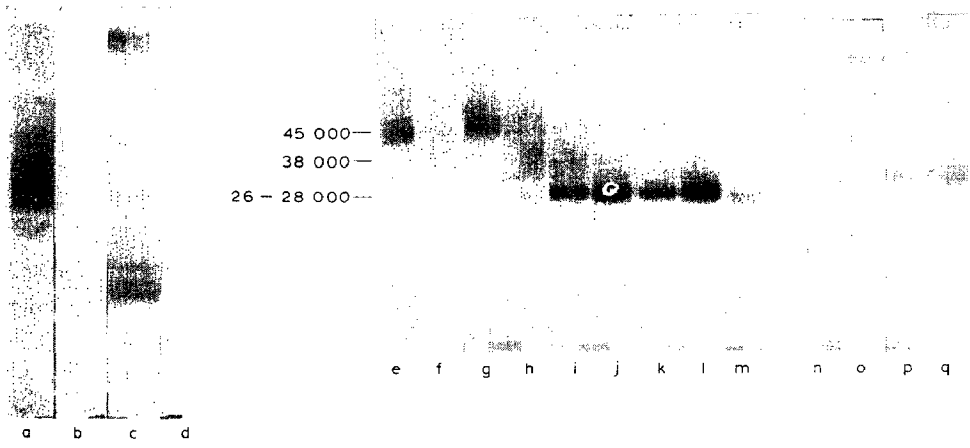

Fig. 3. Effect of endoglycosidases on the Fy^a active components of red cell membranes. Immunoblotting using human anti-Fy^a, prepared as described in the Experimental section, on membranes prepared from cells treated as follows. Tracks (a–f): (a) Fy(a+) cells incubated in the absence of glycosidases; (b) Fy(a−) cells incubated in the absence of glycosidases; (c) Fy(a+) cells incubated with Endo F; (d) Fy(a−) cells incubated with Endo F; (e) Fy(a+) cells incubated in the absence of glycosidases; and (f) Fy(a+) cells incubated with endo-β-galactosidase. Tracks (g–l): Fy(a+) cells (500 μL) incubated for 1 h at 37° with Endo F; (g) none; (h) 0.1 μL; (i) 0.3 μL; (j) 1 μL; (k) 3 μL; and (l) 10 μL. Tracks (m–q): (m) Fy(a+) cells treated with endo-β-galactosidase plus Endo F; (n) Fy(a+) cells incubated in the absence of glycosidases; (o) Fy(a+) cells treated with neuraminidase as described by Anstee *et al.*[11]; (p) Fy(a+) cells treated with Endo F; and (q) neuraminidase-treated Fy(a+) cells, as shown in track (o), further treated with Endo F.

ticular interest because of their possible involvement as receptors for the malarial parasite *Plasmodium vivax*[6,25]. Immunoblotting of membranes from Fy(a+) erythrocytes using human anti-Fy^a antibodies showed a very broad region of binding between M_r 38 000 and 90 000 with the most intensely binding region of M_r 40 000–50 000 (Fig. 3a,b). Treatment with Endo F alone resulted in a dramatic reduction in the M_r of the band to 26 000–28 000 and noticeably sharpened it (Fig. 3c,d). Endo-β-galactosidase treatment of the cells resulted in a reduction in M_r of the leading edge of the band to 33 0000, but the band remained diffuse with the trailing edge spreading to M_r 57 000 (Fig. 3e,f). The Fy^a-binding component was remarkably susceptible to Endo F digestion of intact red blood cells. In an attempt to identify whether intermediate deglycosylated forms of the Fy^a-binding component could be detected, digestion was carried out over a wide range of Endo F concentrations for a short time. Digestion with as little as 1/100 of the standard amount of enzyme for 1 h at 37° caused substantial alterations in the mobility of the Fy^a components. On progressive digestion with Endo F (Fig. 3g–l), two bands of M_r 45 000 and 38 000 appeared. These may represent intermediate deglycosylated forms of the Fy^a component. The combination of Endo F and endo-β-galactosidase reduced the M_r of the band slightly to 25 000 and sharpened it further (Fig. 3m).

These results show that Fy^a-active components are heavily glycosylated. The

Endo F-sensitive carbohydrate is heterogenous and contributes between 40 and 50% of the M_r of the intact molecule. This carbohydrate is, therefore, carried on one or more *N*-glycosyl-linked oligosaccharides. Some, or all, of these oligosaccharides contain poly-*N*-acetyllactosaminyl chains since the M_r of the bulk of the Fy^a antigen component is reduced by endo-β-galactosidase treatment. Treatment of cells with neuraminidase alone reduced the M_r of the leading edge of the Fy^a component by ~4000 daltons, but did not sharpen it (Fig. 3o–q). Hadley *et al.*[25] found a similar reduction in M_r after neuraminidase treatment. When Endo F-treated cells were used, neuraminidase treatment did not cause a further reduction in M_r of the Fy^a component, suggesting that sialic acid-containing, *O*-glycosyl-linked oligosaccharides or other Endo F-resistant oligosaccharides are not present in significant amounts in the Fy^a component. The polydisperse nature of the Fy^a components appears to be due to heterogeniety in the number or composition (or both) of the *N*-glycosyl-linked oligosaccharides in the glycoprotein.

The results presented herein are consistent with the known biochemistry of human red cell surface components. Most of these experiments were carried out by enzyme treatment of intact red cells, and it is likely that, in the case of those components which appear relatively resistant to digestion (for example, sialoglycoprotein β and band 3), more complete degradation would be obtained on treatment of the solubilised proteins under denaturing conditions (Tarentino *et al.*[26]), or if the molecules were modified for example by prior treatment with neuraminidase.

ACKNOWLEDGMENTS

K.R. and N.A. are recipients of MRC Partnership Award Studentships. The authors thank Dr. P. A. W. Edwards for monoclonal antibody R1.3, Dr. S. Moore for monoclonal antibody ES-9, Dr. C. Hodson for human anti-D serum (Dods), Marion E. Reid for suggesting the use of the digitonin–acid method for the preparation of anti-Fy^a for immunoblotting, and Margaret Clark for typing the manuscript and preparing the figures.

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