A New Human Erythrocyte Variant (Ph) Containing an Abnormal Membrane Sialoglycoprotein

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1. A new human erythrocyte variant (Ph) is described. The variant contains an unusual sialic acid-rich glycoprotein in addition to the blood-group-MN(α)- and blood-group-Ss(δ)-active sialoglycoproteins found in normal erythrocytes. 2. The unusual component Ph has an apparent mol.wt. of 32000 on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The Ph component is not degraded during trypsin treatment of intact erythrocytes. 3. The Ph component was labelled by lactoperoxidase-mediated radioiodination of intact erythrocytes and was found to be present in amounts approximately equimolar to α -sialoglycoprotein in the variant erythrocytes. 4. The Ph component had receptors for the lectins from *Maclura aurantiaca* (osage orange) and *Triticum vulgaris* (wheat-germ), but lacked a receptor for the *Phaseolus vulgaris* (red kidney bean) lectin, suggesting that it carries only O-linked oligosaccharides. 5. The presence of the Ph component in these erythrocytes does not correspond to any of the known blood-group-MNSs-related antigens examined. 6. We suggest that this component may be a hybrid polypeptide containing the N-terminal portion derived from normal δ -sialoglycoprotein, and the C-terminal portion from normal α -sialoglycoprotein, in a manner similar to the anti-Lepore haemoglobin.

The human erythrocyte membrane contains two major sialic-acid rich glycoproteins. The sialoglycoprotein α carries the blood-group-MN antigens, whereas the sialoglycoprotein δ [see Anstee *et al.* (1979) for nomenclature] carries the blood-group-Ss antigens in addition to the blood-group-N antigen. Inherited variants of both these sialoglycoproteins are known. Thus homozygous M^k erythrocytes lack both α - and δ -sialoglycoproteins (Tokunaga *et al.*, 1979), whereas M^s and Miltenberger-Class-III erythrocytes contain modified α - and δ -components respectively (Anstee & Tanner, 1978; Anstee *et al.*, 1979). An unusual molecule that appears to be a hybrid of α - and δ -sialoglycoproteins occurs in Miltenberger-Class-V erythrocytes (Anstee *et al.*, 1979). We suggested that this hybrid molecule arises as a result of chromosomal misalignment with unequal crossing-over by a mechanism analogous to that which gives rise to the Lepore haemoglobins. This event results in the loss of the normal α - and δ -components. These variants have all been found in apparently normal healthy blood donors, and it is becoming clear that extensive polymorphism can occur in the α - and δ -sialoglycoproteins without any

significant impairment of the function of human erythrocytes.

During the process of examining erythrocyte samples from individuals heterozygous for the S-s condition (Tanner *et al.*, 1977), one sample was found which gave a markedly abnormal pattern of periodate-staining sialoglycoproteins on SDS/polyacrylamide-gel electrophoresis of the erythrocyte membranes. We have investigated the nature of this abnormality and our results suggest that these variant cells contain an abnormal sialoglycoprotein.

Materials and Methods

Whole blood samples from a Negro individual (M. P. Jr.) and his family were kindly supplied by Dr. R. F. Lowe, Salisbury and District Blood Transfusion Service, Salisbury, Zimbabwe-Rhodesia. S-s- cells (J. N.; Hoekstra *et al.*, 1975) were kindly provided by Mr. G. Newell, Eastern Province Blood Transfusion Service, Port Elizabeth, South Africa.

The methods for the preparation of erythrocyte 'ghosts' and radioiodination of intact erythrocytes have been described previously (Boxer *et al.*, 1974),

Abbreviation used: SDS, sodium dodecyl sulphate.

as have the methods for the location of lectin-binding components (Tanner & Anstee, 1976) and anti-M-antibody-binding components (Anstee *et al.*, 1977). SDS/polyacrylamide-gel electrophoresis was either by the method of Laemmli (1970), with a separating gel containing 10% (w/v) acrylamide with a 3% (tube gels) or a 5% (slab gels) stacking gel, or by the method of Fairbanks *et al.* (1971), using gels containing 8% (w/v) acrylamide. Elution and re-electrophoresis of ^{125}I labelled bands was performed as described by Anstee *et al.* (1979).

Trypsin treatment of intact erythrocytes was carried out as follows: 0.25 ml of washed packed erythrocytes were incubated at 37°C for 30 min with 1 ml of phosphate-buffered saline (0.01 M-sodium phosphate/0.14 M-NaCl), pH 8.0, containing 2.5 mg of trypsin (twice-crystallized, Sigma Chemical Co.) and immediately cooled on ice prior to washing three times with cold phosphate-buffered saline, pH 7.3.

Serological tests were standard direct agglutination procedures. *Vicia graminea* seeds were a gift from Dr. E. A. Steane, Southwestern Medical School, Dallas, TX, U.S.A.

Three rabbits were given intravenous injections of M. P. erythrocytes, the regime described by Ikin (1950) being used.

Results

The original donor (M. P. Jr.) had phenotype MNs and deduced genotype *M-/Ns*. Experiments were done on two separate samples from this donor and on samples from the donor's father (M. P., phenotype MNS-s-, deduced genotype *M-/N-*) and two sisters, Jd. P. and Jl. P. (Fig. 1). Thus M. P. is homozygous for the S-s- condition, whereas M. P. Jr., Jd. P. and Jl. P. are heterozygous for this condition.

SDS/polyacrylamide-gel electrophoresis

The patterns of periodate-staining glycoproteins observed for the erythrocyte membranes from donors M. P. and M. P. Jr. are shown in Fig. 2. Donors Jd. P. and Jl. P. gave the same patterns of staining bands as normal donors. Donor M. P. Jr. gave the periodate-staining components found in normal erythrocytes and a new component of apparent mol.wt. 32000 (denoted Ph in Fig. 2b). In addition, a series of strongly staining bands (denoted αPh , Ph_2 and δPh ; Fig. 2) were obtained in the region between the normal α - and normal α_2 -components. The membranes of donor M. P. gave the same pattern as M. P. Jr., except for the absence of component δ and the band denoted δPh . We interpret these patterns as indicating the presence of an inherited abnormal component (Ph) in erythrocyte membranes of M. P. and M. P. Jr. The relative mobilities of the unusual bands in the sample from M. P. suggest that the band corresponding to

Fig. 1. Family of M. P. Jr., showing the MNSs phenotypes and deduced genotypes

■, Abnormal Ph sialoglycoprotein inherited with the S-s-condition; ▨, S-s-; □, normal; n.t., not tested; the blood-group-MNSs genotypes of the individuals are in italics.

an apparent mol.wt. of 69000 represents the dimer of Ph with itself (Ph_2), whereas the strongly staining bands corresponding to an apparent mol.wt. 76000 represent a mixed dimer of component Ph with component α (αPh). The M. P. Jr. sample contains these components, but in addition contains a component of apparent mol.wt. 59000. This would be the expected molecular weight of a mixed dimer of component Ph and the component δ which is present in these cells (δPh). Both M. P. and M. P. Jr. samples also showed traces of a weakly staining band with an apparent mol.wt. 18000–20000, migrating faster than the normal δ -component. This band was not apparent in Jl. P., Jd. P. or normal control erythrocyte-membrane samples. Trypsin treatment of cells from donor M. P. and subsequent SDS/polyacrylamide-gel electrophoresis of the isolated membranes showed that the bands corresponding to the abnormal Ph monomer and dimer remained unchanged, but the bands corresponding to the α_2 -, αPh - and α -components were removed (results not shown).

Lactoperoxidase radioiodination of M. P. erythrocytes

SDS/polyacrylamide-gel electrophoresis of membranes derived from lactoperoxidase-radioiodinated intact M. P. erythrocytes (Fig. 3) showed that component Ph and its presumed complexes were radioiodinated. In addition, radioactivity was also observed in bands corresponding to apparent

Fig. 2. SDS/polyacrylamide-gel electrophoresis of 'ghosts' from M. P., M. P. Jr. and normal erythrocytes. Separation of 'ghosts' was carried out on tube gels containing 10% (w/v) acrylamide with a 3% overlay, the buffer system of Laemmli (1970) being used. The 'ghost' samples from M. P. and normal erythrocytes were prepared at the same time and 150 μ g of membrane protein was applied to each gel. The 'ghost' sample from M. P. Jr. was prepared on a separate occasion and was not assayed for protein content; 100 μ l of sample was applied to the gel. The gels were stained with the periodic acid/Schiff's-base stain and scanned at 560nm. (a) Normal; (b) donor M. P. Jr.; (c) donor M. P.

mol.wts. 58000, and 18000–20000 (Fig. 3). A 26000-mol.wt. component was also detected on extended radioautography. This labelled 26000-mol.wt. component is found in other S-s- cells (Anstee *et al.*, 1979). The 18000–20000-mol.wt. region was resolved into two bands that correspond with the broad periodate-staining band corresponding to the same molecular weight.

The polypeptide composition of the presumed α_2 , α Ph and Ph_2 bands of M. P. was confirmed by elution and re-electrophoresis of these bands derived from lactoperoxidase-radioiodinated cells (Fig. 4). As expected, the α_2 -band (Fig. 4d) gave components α and α_2 . The α Ph band (Fig. 4e)

yielded α_2 and undissociated α Ph, Ph_2 , α and Ph components. Faint additional bands, corresponding to the 26000-mol.wt. component and its complex with the α -component, were found. This complex migrates in the α Ph region and occurs in S-s-erythrocytes. The Ph_2 band (Fig. 4f) gave Ph and undissociated Ph_2 components. The 58000-mol.wt. component remained largely undissociated on re-electrophoresis, but gave small amounts of the Ph and the 26000-mol.wt. component (Fig. 4a). It is possible that it is a complex of these two components.

The radioactivity in the components obtained on re-electrophoresis of the α Ph-band (Fig. 4e) and the

Fig. 3. SDS/polyacrylamide-gel electrophoresis of membranes from donor M. P. and normal erythrocytes

Electrophoresis was performed on a slab containing 10% (w/v) acrylamide with an overlay of 5% acrylamide in the buffer system of Laemmli (1970). Samples (a) and (b) were stained with the periodic acid/Schiff's base stain. (a) Erythrocyte membranes from donor M. P.; (b) erythrocyte membranes from normal control; (c) and (d) are radioautographs of membranes from radiolabelled cells separated by SDS/polyacrylamide-gel electrophoresis. Packed cells (0.2 ml) were radioiodinated by using 0.4 mCi of ^{125}NaI and 80 μg of lactoperoxidase. (c) Membranes from donor M. P.; (d) membranes from normal control. Abbreviation used: T.D., tracking dye. [The band indicated by the asterisk found in both M. P. and normal control remained substantially undissociated on re-electrophoresis and did not correspond to any of the periodic acid/Schiff's-base staining bands. The band may represent a poorly sialylated membrane component exposed on the outer surface of the cell or possibly a proteolytic digestion product of band 3.]

total radioactivity in the α -containing and Ph-component-containing bands after electrophoresis of labelled membranes (Fig. 3c) was measured. Calculations based on the assumption that the α Ph-band contained equimolar amounts of α - and Ph-components showed that the relative abundance (α/Ph) (donor M. P.) was $1:1.1 \pm 0.2$ (3) in the membrane of M. P. erythrocytes. Similar experiments with normal erythrocytes gave a relative abundance of (α/δ) $1:0.24 \pm 0.05$ (5) for five different normal erythrocytes. The molar proportion of Ph-component compared with α -component in M. P. cells is thus greater than the molar proportion δ/α in normal cells.

Fig. 4. Re-electrophoresis of radiolabelled bands eluted from electrophoretograms of radioiodinated M. P. erythrocytes

Erythrocytes of donor M. P. were radiolabelled as described in the legend to Fig. 3. Membranes from these erythrocytes were separated on a 10% (w/v) acrylamide gel with a 5% overlay (Laemmli, 1970). The wet gel was radioautographed for up to 3 h, and the radioactive bands excised and eluted as described previously (Anstee *et al.*, 1979). (a) Re-electrophoresis of the 58 000-mol.wt. band component (Fig. 3c); (b) whole membranes from donor M. P.; (c) whole membranes from normal control; (d) re-electrophoresis of the Ph_2 band (Fig. 3c); (e) re-electrophoresis of the α/Ph band (Fig. 3c); (f) re-electrophoresis of Ph_2 band (Fig. 3c); (g) as (b); (h) as (c). Abbreviation used: T.D., tracking dye.

Lectin receptors on the abnormal Ph component

Lectin binding experiments showed that Ph and its complexes carried the receptors for the lectins from *Maclura aurantiaca* (osage orange) and *Triticum vulgaris* (wheat germ) but unlike normal α the Ph component does not carry the receptor for *Phaseolus vulgaris* (red kidney bean) lectin (Fig. 5). The *Triticum vulgaris* lectin also bound to the region corresponding to the 58 000-mol.wt. component and to the 18 000–20 000-mol.wt. components.

Serological studies

Agglutination studies showed that members of the family had the MNSs types shown in Fig. 1. M. P. and M. P. Jr. gave normal single-dose reactions for M and N antigens (with four human and two rabbit anti-M sera three human anti-N sera). The cells of M. P. Jr. and J. P. gave positive reactions with one rabbit anti-Hu (Hunter) serum (Landsteiner *et al.*,

Fig. 5. Radioautographs of bound radioiodinated lectins to membranes from donor M. P. and normal erythrocytes, separated by SDS/polyacrylamide-gel electrophoresis

The method for lectin binding has been described previously (Tanner & Anstee, 1976; Anstee *et al.*, 1979). (a and b) Binding of *Phaseolus vulgaris* lectin to: (a) membranes from normal control; (b) membranes from donor M. P. The lectins were radioiodinated as described by Tanner & Anstee (1976). A 1.5 mg sample of labelled lectin with an agglutination titre of 1:128 and containing 10^7 c.p.m. of ^{125}I was used for the gel. (c and d). Binding of *Maclura aurantiaca* lectin to: (c) membranes from donor M. P.; (d) membranes from normal control. The lectins were radioiodinated as described by Tanner & Anstee (1976). Labelled lectin ($100\mu\text{l}$) with an agglutination titre of 1:160 and containing 3.6×10^6 c.p.m. of ^{125}I was used for the gel. (e-g) The binding of wheat (*Triticum vulgaris*)-germ lectin to: (e) membranes from donor M. P.; (f) membranes from donor M. P. Jr.; (g) membranes from normal erythrocytes. A 3.25 mg sample of lectin was radioiodinated (Anstee *et al.*, 1979). The labelled lectin had a titre $>1:2000$ against Pronase-treated human erythrocytes. A 1.6 mg sample, having 1.9×10^7 c.p.m. of ^{125}I , was used for the gel.

Fig. 6. Binding of rabbit anti-M serum to separated components of 'ghosts' from M. P. erythrocytes

The M-antigen-active components were detected by using pig anti-(rabbit immunoglobulin G) as described (Anstee *et al.*, 1977). SDS/polyacrylamide-gel electrophoresis was carried out on gels containing 8% (w/v) acrylamide, $50\mu\text{l}$ of the immunoglobulin G fraction of rabbit anti-M (haemagglutination titre of 1:64 against group-O/MM erythrocytes) and $100\mu\text{l}$ of ^{125}I -labelled pig anti-(rabbit immunoglobulin G) [haemagglutination titre 1:160 when tested by the direct anti-globulin method against human erythrocytes coated with a rabbit anti-(human erythrocyte) serum] containing 1.6×10^8 c.p.m./ml were used. The scan shows the densitometer trace at 500 nm of a radioautograph of a dried slice of the gel.

1934); Jd. P. reacted with anti-(Tm + Sj) serum (Issitt *et al.*, 1968). The presence of these antigens is not associated with the Ph-component, since the cells of M. P. do not carry them. Agglutination tests on cells from the family members showed that the following MNSs-related antigens are not associated with the abnormal Ph component: M₁, M^s, M^v, Mt^a, He, Sul, Ny^a, Cl^a, Ri^a, St^a, Mi^a, Vw, Mur, Hil, Lane and Vr. Intravenous injection of three rabbits with the erythrocytes of M. P. resulted in the production of only anti-M and weak anti-N antibodies. No antibodies were obtained that reacted specifically with the erythrocytes of M. P., or M. P. Jr.

Unlike trypsin-treated normal erythrocytes, trypsin-treated S-s- erythrocytes are not agglutinated by *Vicia graminea* lectin (Dahr *et al.*, 1975). However, trypsin-treated M. P. erythrocytes, although they are S-s- (Fig. 1), reacted strongly with the *Vicia graminea* lectin. Trypsin-treated normal erythrocytes were agglutinated with a titre of 1:256, whereas trypsin-treated M. P. cells reacted with a titre of 1:64 as did trypsin-treated Jd. P. cells (a S-s-heterozygote). In the same experiment, no reaction was observed with trypsin-treated J. N. cells (a homozygous S-s- donor; Hoekstra *et al.*, 1975). These results suggest that the Ph-component contains a receptor for the *Vicia graminea* anti-N lectin and thus carries N-antigen activity.

Since both M. P. and M. P. Jr. have blood-group-M activity, we decided to determine whether this activity was located on normal α - or on the abnormal Ph-component. Anti-M-antibody-binding experiments (Fig. 6) showed that the antibody bound to the α_2 -, α Ph- and α -bands, and strong binding was observed in the region of the 18000–20000-mol.wt. band detected by periodic acid/Schiff's base staining (Fig. 2). However, the conditions of electrophoresis used in this experiment did not allow the positive assignment of this binding to the 18000–20000-mol.wt. periodate-staining component (Fig. 2). Since all the α -component-containing bands bound anti-M antibody, whereas the Ph and Ph₂ bands did not, we conclude that the M-antigen activity of M. P. and M. P. Jr. is located on the normal α -component. In addition, trypsin treatment of M. P. erythrocytes resulted in the loss of M-antigen as detected by direct agglutination tests with a human anti-M serum. This result is also consistent with the interpretation that the M-antigen activity is located on the normal α - rather than the Ph-component, which resists trypsin digestion in these cells.

Discussion

Our results clearly show the presence of an abnormal component (Ph) in the cells of M. P. and M. P. Jr. The apparent molecular weight of the Ph

component is 32000 on a 10% (w/v) acrylamide gel and it forms complexes with both α - and δ -components. The lectin-binding characteristics of the Ph component suggest it carries oligosaccharides similar to those found on α - and δ -components, since it binds both *Maclura aurantiaca* and *Triticum vulgare* lectins. The binding of *Triticum vulgare* lectin to the Ph-component does not necessarily indicate the presence of an N-glycosidically linked oligosaccharide, since the lectin binds to normal δ -component, which lacks this type of oligosaccharide (Furthmayr, 1978). In this case, sialic acid may be responsible for the binding of *Triticum vulgare* lectin to δ -component (Greenaway & LeVine, 1973; Adair & Kornfeld, 1974). However, component Ph lacks the *Phaseolus vulgaris* receptor that is found on normal α but that is absent from the δ -component. This lectin is thought to bind to the N-glycosidically linked oligosaccharide present in the normal α -component (Kornfeld & Kornfeld, 1971). The Ph component shares the characteristic resistance to trypsin and reactivity with the *Vicia graminea* lectin (and thus N-antigen activity) of the δ -component.

The inheritance of M-antigen activity in M. P. and M. P. Jr. is linked with the inheritance of the Ph-component (Fig. 1). However, the M-antigen activity is present on normal α -component and not on the Ph-component, which carries N-antigen activity. The co-inheritance of α - and Ph-components shows that component Ph does not simply arise from an allele of normal α -component. Since the cells of M. P. totally lack Ss-antigen activity (and normal δ -component), it is possible that component Ph arises from an unusual allele of the δ -component: one that lacks Ss antigens. An unusual allele of δ -component occurs in Miltenberger-Class-III erythrocytes, but this carries s-antigen activity (Anstee *et al.*, 1979).

However, M. P. erythrocytes contain approximately equimolar amounts of the α - and Ph-components (approximately four times more abundant than δ -component). Furthermore, component Ph differs from component δ in two respects, in that although its apparent molecular weight is greater than that of component δ , it has lost Ss-antigen activity. Such a structure would require two independent genetic events to evolve from component δ , and there would also need to be a change in the amounts incorporated in the membrane. This suggests that component Ph does not arise simply from an unusual allele of the δ -gene.

The possibility exists that component Ph is a hybrid molecule containing elements derived from α - and δ -components. The abnormal sialoglycoprotein found in Miltenberger-Class-V (Mi.V) erythrocytes appears to be a hybrid molecule derived from α - and δ -components (Anstee *et al.*, 1979). We

Fig. 7. Possible origin of abnormal sialoglycoprotein Ph in the family of M. P. Jr.

M, N, S and s refer to blood-group antigens characteristic of the sialoglycoproteins. N and C denote the N-terminus and C-terminus of individual sialoglycoproteins; NT and CT refer to the N-terminal portion and C-terminal portion of components α and δ .

have suggested that the abnormal component in Mi.V erythrocytes is derived in a manner analogous to the Lepore haemoglobins by chromosomal misalignment and unequal crossing-over between α - and δ -genes to yield a molecule with the N-terminus derived from component δ [denoted $(\alpha-\delta)^{Mi.V.}$] and the concomitant loss of the normal α - and δ -components. If component Ph is indeed a hybrid molecule containing elements derived from both α - and δ -components, then it is likely to contain the N-terminal sequence of component δ and the C-terminal sequence of component α . The resistance of the Ph component to cleavage by trypsin and its lack of a *Phaseolus vulgaris* lectin receptor suggests that the N-terminus of the molecule is not derived from component α . These properties are characteristic of component δ , as is the occurrence of N-antigen activity. It is possible that the similar abundance of components Ph and α may reflect the presence of a region derived from α in the C-terminus of component Ph. The C-terminal portion of the sialoglycoproteins may influence the number of molecules inserted into the membrane, since this region is responsible for the binding of these proteins to the membrane.

Unlike the α - δ hybrid molecule found in Mi.V erythrocytes, component Ph appears to be a δ - α hybrid $[(\delta-\alpha)^{Ph}]$. This arrangement of δ - and α -portions of the $(\delta-\alpha)^{Ph}$ component could arise by chromosomal misalignment, with unequal crossing-

over, in a manner analogous to the formation of 'anti-Lepore'-type haemoglobins (Bunn *et al.*, 1977). In the 'anti-Lepore' situation one would expect the presence of both normal α - and δ -components in the erythrocyte membrane in addition to the 'anti-Lepore' hybrid. Since the presence of M-antigen activity and lack of Ss-antigen activity is inherited with the $(\delta-\alpha)^{Ph}$ component in the cells of M. P. and M. P. Jr., the event resulting in the formation of the 'anti-Lepore' would have to have occurred between a normal chromosome and one that does not express the normal δ character (Fig. 7). The S-s- phenotype that results from the lack of expression of the normal δ character is relatively common in Negroid populations (Lowe & Moores, 1972).

Although our results suggest that component $(\delta-\alpha)^{Ph}$ results from an 'anti-Lepore'-type event, confirmation that this is indeed the case must await detailed structural analysis. Component $(\delta-\alpha)^{Ph}$ is a further example of an abnormal α - and δ -related sialoglycoprotein found in a healthy blood donor. This discovery extends the range of detected polymorphisms of α - and δ -components that apparently have no deleterious effects on erythrocyte function.

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