A Method for the Direct Demonstration of the Lectin-Binding Components of the Human Erythrocyte Membrane

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1. A method which allows the characterization of lectin-binding components is described. This method should be useful in defining the nature and heterogeneity of these components in cell membranes. 2. The method, which we have used on erythrocyte 'ghosts', involves the fixation of 'ghost' components after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and incubation with purified ^{125}I -labelled lectins. 3. Each of the four lectins used shows an individual pattern of reactivity towards 'ghost' components. Band 3, the major membrane-penetrating glycoprotein, is bound by the lectins from *Ricinus communis* and *Phaseolus vulgaris* (phytohaemagglutinin) and by concanavalin A. The major erythrocyte sialoglycoprotein is bound by the lectins from *R. communis*, *P. vulgaris* and *Maclura aurantiaca*. 4. Three of the lectins display binding for other membrane components, some of which are not demonstrable by conventional protein- and carbohydrate-staining techniques.

The structure and function of cell-surface glycoproteins has been the subject of intensive studies (Oseroff *et al.*, 1973). A frequently adopted approach to this problem has involved the use of specific carbohydrate-binding proteins (lectins) of both plant and animal origin (Lis & Sharon, 1973). The interpretation of this type of experiment is sometimes complicated by difficulties in defining the particular carbohydrate-containing components to which the lectins bind (Lis & Sharon, 1973). A method which allows the characterization of lectin-binding components would be potentially valuable to help define the nature and heterogeneity of such components in cell membranes. It would also be a useful technique with which to study minor carbohydrate-containing components which are often not readily detected by conventional protein- or carbohydrate-detection methods.

In the present paper we investigate the binding components of the human erythrocyte membrane for four lectins with the use of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to separate the 'ghost' components.

Materials and Methods

The preparation of erythrocyte 'ghosts' and methods for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were as described by Boxer *et al.* (1974).

Preparation of labelled lectins

The lectins were labelled with ^{125}I (carrier-free; The Radiochemical Centre, Amersham, Bucks.,

U.K.) by the chloramine-T method (Klinman & Taylor, 1969).

Concanavalin A. Concanavalin A (Pharmacia, Uppsala, Sweden) (10mg) was dissolved in 1ml of iso-osmotic sodium phosphate buffer, pH 7.4, containing 0.3M-methyl α -D-mannoside [Sigma (London) Chemical Co., London S.W.6, U.K.]. The protein was iodinated with 1 mCi of ^{125}I and 200 μg of chloramine-T (BDH Chemicals, Poole, Dorset, U.K.). After labelling, 10mg of bovine haemoglobin (Sigma) was added as carrier. The solution was exhaustively dialysed against phosphate-buffered saline (9vol. of 0.15M-NaCl added to 1 vol. of iso-osmotic sodium phosphate buffer, pH 7.4) at 4°C. The labelled protein was repurified by application to a column (1 cm $\times$ 25 cm) of Sephadex G-50 (fine) equilibrated with phosphate-buffered saline, and eluted with 0.2M-methyl α -D-mannoside in phosphate-buffered saline. Bovine haemoglobin (10mg) was added before exhaustive dialysis against phosphate-buffered saline at 4°C. The agglutination titre of the lectin against Pronase-treated blood-group-O human erythrocytes was not significantly affected by the labelling procedure. The final preparation contained 7×10^6 c.p.m./mg of protein.

P. vulgaris lectin. Lectin (Difco, Detroit, Mich., U.S.A.; type P) (10mg) was labelled in the presence of 0.3M-N-acetyl-D-galactosamine (Sigma), as described for concanavalin A, but the protein was not repurified after labelling. The haemagglutination titre of this lectin against blood-group-O human erythrocytes fell from 1:128 (5mg of lectin/ml) before labelling to 1:8 (5mg of lectin/ml) after labelling. The final preparation contained 1×10^7 c.p.m. of ^{125}I /mg of protein.

R. communis lectin. *R. communis cambodgensis* seeds (Thompson and Morgan, Ipswich, Suffolk, U.K.) (2g) were homogenized in 20ml of phosphate-buffered saline. After centrifugation at 900g for 10min, the supernatant was filtered through Whatman no. 1 paper. The lectin was further purified by absorption on and elution from formaldehyde-treated human erythrocytes as described by Reitherman *et al.* (1974). A portion (2ml) of the filtrate was agitated with 6ml of formaldehyde-treated human blood-group-O erythrocytes (Lockyer *et al.*, 1967) and 10ml of phosphate-buffered saline on a rotary mixer for 30min at room temperature (21°C). The cells were washed in the centrifuge with phosphate-buffered saline until the supernatant was free of protein. Phosphate-buffered saline containing 0.3M-D-galactose (12ml) was added to the cells, and the suspension mixed for 30min. The cells were removed by centrifugation at 900g for 10min, and the supernatant was exhaustively dialysed against distilled water at 4°C. The protein was freeze-dried, and the dried material dissolved in 0.5ml of iso-osmotic sodium phosphate buffer, pH7.4, for labelling with 1mCi of ^{125}I and 5 μg of chloramine-T. Bovine haemoglobin (30mg) was added, and the labelled lectin exhaustively dialysed against phosphate-buffered saline at 4°C. The haemagglutination titre was 1:64. The final labelled solution contained 1.6×10^7 c.p.m. of ^{125}I /ml.

M. aurantiaca lectin. *M. aurantiaca* seeds (Thompson and Morgan, Ipswich, Suffolk, U.K.) (2.5g) were homogenized in 25ml of phosphate-buffered saline. The suspension was centrifuged at 900g for 10min and the clear supernatant removed without contamination by the floating lipid layer. The lectin was purified from 2ml of this supernatant by absorption on and elution from formaldehyde-treated cells as described for *R. communis* lectin, except that the adsorption mixture contained 2ml of formaldehyde-treated human blood-group-O erythrocytes and 2ml of phosphate-buffered saline. The lectin was eluted with 4ml of phosphate-buffered saline containing 0.3M-D-galactose. The dialysed freeze-dried lectin was dissolved in 2ml of iso-osmotic phosphate buffer, pH7.4, for labelling with ^{125}I . This material had an agglutination titre of 1:1024 against normal human blood-group-O erythrocytes. The lectin solution was labelled in the presence of 0.3M-D-galactose with 1mCi of ^{125}I and 200 μg of chloramine-T. Bovine haemoglobin (10mg) was added before exhaustive dialysis against phosphate-buffered saline at 4°C. The labelled lectin was repurified by adsorption on a column (1cm $\times$ 15cm) of Sepharose 6B (Pharmacia) equilibrated with phosphate-buffered saline and eluted with phosphate-buffered saline containing 0.2M-D-galactose. The size of this column was inadequate since approx. 75% of the lectin haemagglutination activity was not retained. After exhaustive dialysis against phosphate-buffered saline at 4°C,

the solution (11ml) had a haemagglutination titre of 1:8 and contained 7×10^4 c.p.m. of ^{125}I /ml.

Reaction of labelled lectins with samples of human erythrocyte 'ghosts' separated by gel electrophoresis

Samples of erythrocyte 'ghosts' were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on 1.1cm diam. gels containing 8% (w/v) acrylamide. The gels were sliced longitudinally and two flat centre slices taken for reaction with labelled lectin as described below. The curved slices from the edge of the gel were stained with the periodic acid-Schiff's-base stain to locate the carbohydrate-staining components. The central slices were fixed by a method based on that of Keck *et al.* (1973). Each slice was placed in 100ml of 50% (v/v) methanol for 30min; 0.2ml of 25% (w/v) glutaraldehyde (BDH) was added, and the slices were left for 1.5h. They were then removed and placed in a mixture of 100ml of 0.15M-NaCl and 50ml of 0.1M-sodium phosphate buffer, pH8.0. The solution was made 20 μg /ml in NaBH_4 by addition of the solid. After 1h the solution was decanted and the slices were left overnight in 150ml of the same NaBH_4 -containing solution. They were then soaked in two or three washes of 150ml of phosphate-buffered saline, the wash being changed every 2-3h. The slices were transferred to 20ml vials containing 4ml of phosphate-buffered saline, 2mg of bovine haemoglobin carrier/ml and 0.05% sodium azide. The labelled lectin and appropriate monosaccharide inhibitor (if applicable) were added and the mixture was shaken gently for 3 days. The solution was decanted and the gel slices were washed for 3 days in 200ml of phosphate-buffered saline containing 0.05% sodium azide and the appropriate monosaccharide inhibitor (if applicable). The slices were then stained for protein with Coomassie Blue and dried down together with the side slices which had been stained with the periodic acid-Schiff's-base stain. The labelled bands were detected by radioautography. The radioautographs and dried protein-stained gels were scanned in a model G Scanner (Canalco, Rockville, Md., U.S.A.).

Results and Discussion

The reactions of a large number of lectins with the human erythrocyte surface have been extensively studied (see Sharon & Lis, 1972). However, in most cases it has not been possible to define unambiguously the individual membrane components which are involved in these reactions. In this paper we describe a simple method for the direct demonstration of the binding of lectins to separated membrane components. Human erythrocyte 'ghost' components after separation by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis can be fixed *in situ* by glutaraldehyde treatment. Gels so fixed can be stored for at

least 3 weeks without the loss of any protein-staining component. Slices of gels fixed in this way were incubated with ^{125}I -labelled purified lectin and after washing were stained and dried. The labelled bands were detected by radioautography. As far as possible, reaction in the presence of the appropriate monosaccharide inhibitor was used as a control for non-specific binding of the lectin. The nomenclature for the human erythrocyte membrane components adopted in this paper is that of Steck (1974). Two major glycoproteins (band 3 and band PAS 1) are accessible on the outer surface of the human erythrocyte membrane together with three or four minor glycoproteins (Steck, 1974; Mueller & Morrison, 1974). It is thought that all of the carbohydrate of the human erythrocyte membrane is at the extracellular surface (Steck, 1974).

Binding of R. communis lectin to human erythrocyte membrane components

Fig. 1 shows the pattern of binding of the *R. communis* lectin to human erythrocyte membrane components. The lectin binds to band 3 and band PAS 1 (the major erythrocyte sialoglycoprotein). A broad area of labelled lectin is also found between bands 4.2 and 5. This lectin is known to bind prefer-

entially non-reducing galactosyl residues (Olsnes *et al.*, 1974; Nicolson *et al.*, 1974) and the binding of the labelled lectin was completely abolished by the addition of 0.2M-D-galactose in the reaction mixture and 0.1M-D-galactose in the wash solution (Fig. 1).

Adair & Kornfeld (1974) did not find component PAS 1 among the *R. communis* receptor components which they obtained from the human erythrocyte membrane. However, our results suggest that this lectin binds component PAS 1. Dahr *et al.* (1975) have provided evidence by the use of haemagglutination-inhibition experiments that isolated component PAS 1 has *R. communis* receptor activity, and we have obtained similar results with component PAS 1 purified as described by Anstee & Tanner (1975). However, Adair & Kornfeld (1974) used deliberately overloaded affinity columns. The discrepancy may therefore reflect a lower binding affinity of component PAS 1 for the lectin column when compared with the other membrane components isolated.

This lectin shows areas of strong binding in the region between bands 4.2 and 4 which are not clearly associated with any of the membrane components which can be detected by protein or carbohydrate stains. Steck & Dawson (1974) and Gahmberg & Hakomori (1973) found extensive labelling in this region using the galactose oxidase method. Our results confirm the presence of galactose-containing moieties in this area. The nature of these components has not been clearly defined. It is possible that they are glycoproteins, or alternatively, it may be that they represent complex glycolipids containing large oligosaccharide chains, such as the megaloglycolipids suggested by Gardas & Koscielak (1974).

Binding of P. vulgaris lectin (phytohaemagglutinin) to human erythrocyte 'ghost' components

The pattern of binding of this lectin to separated human erythrocyte 'ghost' components is shown in Fig. 2. The major binding components correspond to band 3 and the erythrocyte sialoglycoprotein (PAS 1). In addition, the region in the area of band PAS 2 is strongly labelled. An area of diffuse labelling is found between bands PAS 1 and PAS 2. Pretreatment of the cells with trypsin resulted in loss of the band corresponding to component PAS 1 and some of the material around band PAS 2 (Fig. 2). *N*-Acetyl-D-galactosamine is a weak inhibitor of this lectin (Pusztai & Watt, 1974), but the high cost of the compound precluded its use as an inhibitor in this system. When 0.1M-*N*-acetyl-D-galactosamine was included in the reaction mixture but omitted from the wash solution no significant change in the pattern of labelling was detected. However, the addition of formaldehyde-stabilized human group-O erythrocytes to the reaction mixture abolished the binding of the labelled lectin.

Fig. 1. Binding of the radioiodinated lectin from *R. communis* to human erythrocyte 'ghost' components

'Ghosts' from blood-group-A erythrocytes were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The two centre slices were incubated with 0.05 ml of *R. communis* lectin (containing 8×10^5 c.p.m. of ^{125}I) as described in the Materials and Methods section. D-Galactose (0.2M) was present in the reaction mixture of one slice as a control, and this was also washed in the presence of 0.1M-D-galactose. (a) Scan of protein stain (Coomassie Blue) of dried gel. (b) Scans of radioautographs of dried gels. —, Incubation in the absence of D-galactose; ----, incubation in the presence of D-galactose.

Fig. 2. Binding of the radioiodinated lectin from *P. vulgaris* to human erythrocyte 'ghost' components

'Ghosts' from human blood-group-A erythrocytes and from trypsin-treated blood-group-A erythrocytes were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The centre slices were incubated with 0.25 mg of labelled *P. vulgaris* lectin (containing 2.5×10^6 c.p.m. of ^{125}I) as described in the Materials and Methods section. (a) Scan of protein stain (Coomassie Blue) of dried gel of untreated erythrocyte 'ghosts'. (b) Scans of radioautographs of dried gels. —, Untreated human erythrocyte 'ghosts'; ----, trypsin-treated human erythrocyte 'ghosts'.

Fig. 3. Binding of the radioiodinated lectin from *M. aurantiaca* to human erythrocyte 'ghost' components

'Ghosts' from blood-group-A erythrocytes were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Two slices were taken from the centre of the gel and each was incubated with 0.5 ml of the labelled lectin from *M. aurantiaca* (containing 3.5×10^6 c.p.m. of ^{125}I) as described in the Materials and Methods section. D-Galactose (0.2 M) was present in the reaction mixture (and 0.1 M-D-galactose present in the wash solution) of one of the gel slices to serve as a control. (a) Scan of protein stain (Coomassie Blue) of dried gel. (b) Scan of radioautographs of dried gels. —, Incubation in the absence of D-galactose; ----, incubation in the presence of D-galactose.

Binding of *M. aurantiaca* (syn. *Maclura pomifera*) lectin to human erythrocyte 'ghost' components

The pattern of binding of this lectin to separated human erythrocyte 'ghost' components is shown in Fig. 3. The protein binds only to components PAS 1, PAS 2 and PAS 3. The binding of the labelled lectin was completely abolished by the presence of 0.2 M-D-galactose in the reaction mixture and 0.1 M-D-galactose in the wash solution (Fig. 3).

Components PAS 1, PAS 2 and PAS 3 probably react with the periodic acid-Schiff's-base stain because they contain large amounts of sialic acid. The major sialic acid-containing component of band PAS 1 is the alkali-labile tetrasaccharide isolated by Thomas & Winzler (1969) and Adamany & Kathan (1969), and it has been suggested that this oligosaccharide is the receptor for *M. aurantiaca* lectin in component PAS 1 (Dahr *et al.*, 1975). The similar reactivity of components PAS 1, PAS 2 and PAS 3 observed here suggests the possibility that a similar oligosaccharide unit may be present in each of these glycoprotein bands.

Binding of *C. ensiformis* lectin (concanavalin A) to human erythrocyte 'ghost' components

Fig. 4 shows the binding of this lectin to separated human erythrocyte 'ghost' components. The lectin binds primarily to band 3, and this binding was

Fig. 4. Binding of radioiodinated concanavalin A to human erythrocyte 'ghost' components

'Ghosts' from blood-group-A erythrocytes were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Two slices were taken from the centre of the gel and each was incubated with 0.16 mg of radioiodinated concanavalin A (containing 1.1×10^6 c.p.m. of ^{125}I) as described in the Materials and Methods section. Methyl α -D-mannoside (0.2 M) was present in the reaction mixture (and 0.2 M-D-glucose was present in the wash solution) of one of the slices to serve as a control. (a) Scan of protein stain (Coomassie Blue) of dried gel. (b) Scans of radioautographs of dried gels. —, Incubation in the absence of methyl α -D-mannoside; ----, incubation in the presence of methyl α -D-mannoside.

Table 1. *Reactivity of lectins with erythrocyte membrane components*

+ indicates binding of labelled lectin to membrane component; – indicates absence of binding.

Source of lectin	Sugar specificity	Erythrocyte components		
		Band 3	PAS 1	Region 4.2–5
<i>R. communis</i>	Galactose	+	+	+
<i>P. vulgaris</i>	<i>N</i> -Acetylgalactosamine	+	+	+
<i>M. aurantiaca</i>	Galactose/ <i>N</i> -acetylgalactosamine	–	+	–
<i>C. ensiformis</i>	Glucose/mannose/fructose/ <i>N</i> -acetyl-D-glucosamine	+	–	–

abolished by the addition of 0.2M-methyl α -D-mannoside to the reaction mixture and 0.2M-D-glucose to the wash solution. The level of background labelling tended to be higher with concanavalin A than with other lectins. Surprisingly, this background labelling was also decreased by the presence of the monosaccharides. It is not likely that this background reflects the specific binding of concanavalin A since bands 1, 2, 5 and 6, which predominate among the background labelling, are known to be situated at the cytoplasmic surface of the membrane and all the membrane carbohydrate is believed to be on the outside of the membrane (Steck, 1974). There is no evidence for the presence of carbohydrates on any of these components. Pretreatment of the cells with trypsin, under conditions which degrade component PAS 1, did not affect the observed pattern of binding. It is noteworthy that the binding of concanavalin A was primarily associated with the front edge of the protein 3 band. This suggests that not all the material in band 3 can bind to concanavalin A. Findlay (1974) has found that only a small proportion of the material in band 3 is bound to concanavalin A/Sephacrose columns. It is possible that this may be a result of heterogeneity in the carbohydrate present on the protein.

The results are summarized in Table 1. Although the lectins from *R. communis* and *P. vulgaris* bind to carbohydrate associated with band 3 and band PAS 1, the oligosaccharides associated with these two membrane glycoproteins can be distinguished by their different reactivities with the lectin from *M. aurantiaca* and with concanavalin A. The *M. aurantiaca* lectin binds to the carbohydrate associated with band PAS 1 but not that of band 3. In contrast, concanavalin A binds only to band 3 but not band PAS 1.

At least part of the material in the band described as PAS 2 appears to be an alternative form of component PAS 1 (Tuech & Morrison, 1974; Mueller & Morrison, 1974; Tanner & Anstee, 1976), and therefore it would be expected that any lectin that binds component PAS 1 should also bind this component in the PAS 2 band. Although this is clearly the case

with the lectin from *M. aurantiaca*, the extensive labelling due to other components in this area makes it difficult to establish that the lectins from *R. communis* and *P. vulgaris* also bind this component. The only lectin that clearly binds to component PAS 3 is that of *M. aurantiaca*.

Lectins specific for all the sugars commonly found in cell surface membranes are available (Sharon & Lis, 1972). In principle it should be possible to utilize this method and the different specificities of a number of lectins to obtain information about the composition of the carbohydrate moieties present in the individual components of complex mixtures of proteins such as those that are present in cell membranes. Similarly, it should be possible to obtain quantitative estimates of the amounts of the individual components in such mixtures.

It is clear that in many cases lectins bind to a number of distinct membrane components in the human erythrocyte. This is also likely to be the case for the lectin-binding components of other cell membranes. It is of importance to define the nature and heterogeneity of lectin receptors when studying the reactivity of lectins with intact cells, and the method described here is likely to be a useful technique for this purpose.

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