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IMMUNOABSORPTION OF MEMBRANE-SPECIFIC ANTIBODIES FOR DETERMINATION OF EXPOSED AND HIDDEN PROTEINS IN HUMAN ERYTHROCYTE MEMBRANES*

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SUMMARY

Human erythrocyte membrane proteins solubilized with the non-ionic detergent Berol EMU-043 have been characterized by crossed immunoelectrophoresis with rabbit antibodies raised against the membrane material. Three out of sixteen membrane-specific immunoprecipitates disappeared when the antisera were first absorbed with intact erythrocytes. This finding indicates that three antigens are exposed on the outside of the erythrocyte membrane. One of these antigens showed acetylcholinesterase activity, and another was the major glycoprotein (glycophorin) as shown by crossed-line immunoelectrophoresis. No antigenic determinants of the latter protein were detected within the membrane or on its inner surface.

In crossed immunoelectrophoresis with antisera after absorption with washed, non-sealed membranes only one precipitate remained. This precipitate corresponded to albumin. Accordingly, several proteins seem to have antigenic determinants exposed on the inside of the membrane.

INTRODUCTION

In a search for new methods to study membrane proteins, human erythrocyte membranes have been extensively solubilized with a non-ionic detergent and the antigen solution has been characterized by crossed immunoelectrophoresis using a rabbit antibodies which precipitated most of the solubilized proteins [1]. In the present study we show that the proteins corresponding to certain of the precipitates can be identified by immunoelectrophoretic comparison with the purified proteins, or by the catalytic activity of the precipitate. Furthermore, selective removal of antibodies by absorption with intact cells or with washed, non-sealed membranes can be analyzed by crossed immunoelectrophoresis using the depleted antibody solution and is revealed

* Part of this work was presented at the 8th FEBS Meeting (1972) in Amsterdam [4].

by the disappearance of the precipitation lines corresponding to the exposed antigens of the absorbing membrane. One can thus learn whether an antigenic membrane protein is exposed on the inside or the outside of the membrane [2, 3]. This technique has been used to localize membrane proteins from *Acholeplasma laidlawii* [3] and is here applied to human erythrocyte membranes.

MATERIALS AND METHODS

Membrane material

The erythrocytes in outdated citrated human blood were washed repeatedly in autologous plasma (4 times at 600 x *g* for 20 min). The erythrocytes were contaminated with less than one leucocyte per million erythrocytes as shown by lysis, staining with 0.02 % (w/v) Gentian Violet and counting in a Bürker-Türk counting chamber (Carl Zeiss, Jena, G.F.R.). The erythrocytes were then repeatedly washed in phosphate buffer and membranes were prepared according to Dodge et al. [5]. The final membrane suspension contained small amounts of hemoglobin. The total protein concentration was 4 g/l as determined by the method of Lowry [6]. Membranes not used immediately were stored at – 20 °C. The membranes were extensively solubilized in 0.0038 M Tris and 0.010 M glycine, pH 9.2 (5 °C) containing 1 % (w/v) Berol EMU-043 (MoDo Kemi, Stenungsund, Sweden) as described in ref. [1]. The major glycoprotein (the MN-glycoprotein, glycophorin) was prepared according to Marchesi [7] except that the membranes had first been extracted at 20 °C for three times one day with water at low ionic strength (5 mM EDTA, pH 7.4) to remove mainly spectrin (cf. refs 8–11). The preparation contained 2 g of protein per l and showed mainly one protein zone when subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate [12].

Antibodies

Antibodies against human erythrocyte membrane material were produced and purified as described earlier [1]. The antibody preparation contained anti-albumin but no precipitating antibodies against any other serum protein. The concentration of immunoglobulins was 30 g/l.

Absorption of antibodies with intact erythrocytes

Freshly drawn heparinized blood was centrifuged and the erythrocytes were washed five times in 0.154 M NaCl. 10 ml of erythrocytes packed by centrifugation at 250x*g* for 15 min were gently mixed at 37 °C with 1 ml (30 mg) of antibodies and 6 ml of 0.154 M NaCl. The erythrocytes agglutinated upon addition of the antibodies. After 30 min of gentle shaking at 37 °C the mixture was centrifuged at 250x*g* for 15 min. Hemolysis was negligible. The cell volume of the packed erythrocytes was about 7 ml as determined by dilution experiments with Blue Dextran 2000 (Pharmacia, Uppsala, Sweden). Accordingly, an antibody solution not depleted by absorption but diluted with 9 vol of 0.154 M NaCl was used as a control in the immunoelectrophoretic experiments. Antibody solutions that had been depleted twice by absorption as above gave identical results. In some immunoelectrophoresis experiments the antibody solution was first dialyzed against the immunoelectrophoresis buffer, which led to sharper precipitation lines.

Absorption of antibodies with washed, isolated membranes

The antibody solution was mixed with membranes prepared according to Dodge et al. [5] in 8 mM phosphate buffer pH 7.4 (2.5 g of immunoglobulin per g of membrane protein) and was gently shaken for 30 min at 37 °C. The antibodies in the supernatant after centrifugation at 41 000x*g* for 1 h were used for crossed immunoelectrophoresis of Berol-EMU-043-solubilized membrane material.

Immunoelectrophoresis

Crossed [13] and crossed-line immunoelectrophoresis [14] of the Berol-EMU-043-solubilized membrane material were performed at 16 °C in 1 % (w/v) agarose (Litex, Glostrup, Denmark, batch 102) gel containing 0.038 M Tris and 0.10 M glycine (pH 8.9) and 1 % (w/v) Berol EMU-043 as described in [1]. Some experiments were performed without detergent in the gel. Crossed immunoelectrophoresis with an intermediate gel was performed as described by Axelsen [15] with 1.5 ml of antibody solution depleted by absorption with cells or membranes in the intermediate gel strip.

After electrophoresis the plates were pressed, washed and dried, and finally stained with Coomassie Brilliant Blue R 250, all as described in [16]. The amount of albumin was determined by measuring the area below the precipitate with a planimeter and comparing with the areas obtained with various amounts of pure human albumin (reinst, Behringwerke AG, Marburg/Lahn, G.F.R).

Staining for esterase activity

A staining solution [17] (modified after Stern and Lewis [18]) was prepared by mixing 25 mg of 5-chloro-*o*-toluidine (Fast Red TR) with 1 ml of 1 % (w/v) 1-naphthyl acetate in acetone (both reagents from Sigma, St. Louis, Mo., U. S. A.), and with 50 ml of 0.2 M sodium phosphate buffer (pH 7.4).The agarose gels were washed, pressed, and dried at room temperature and were then kept for 1 h at 30 °C in the above solution. Esterase activity gives rise to a brown-red precipitate. The reaction was totally inhibited by 10^{-5}M eserine (physostigmine) which indicates that the enzyme was a cholinesterase [19], probably acetylcholinesterase (EC 3.1.1.7), which is the only known cholinesterase in the human erythrocyte membrane [20]. Finally the gels were rinsed in water for 1 h, dried, and stained in the ordinary way with Coomassie Brilliant Blue R 250 [16].

RESULTS

Non-depleted (normal) antibody solution

Crossed immunoelectrophoresis of Berol-EMU-043-solubilized proteins from human erythrocyte membranes gave the pattern shown in Fig. 1A. The precipitates can be recognized by their relative positions, staining intensity and shape. In Fig. 1A precipitates 2, 16, 17 and 19 are numbered as in refs [1] and [21]. In addition, a precipitate with cholinesterase activity and a precipitate not seen previously have been numbered 20 and 21, respectively. However, precipitate 21 might correspond to that previously numbered 15, which could not be identified in the present experiments. Nor were precipitates3 and 4 in refs 1 and 21 observed with the antibody solution used.

Antibody solution depleted with intact erythrocytes

After the antibody solution had been treated with intact erythrocytes to remove

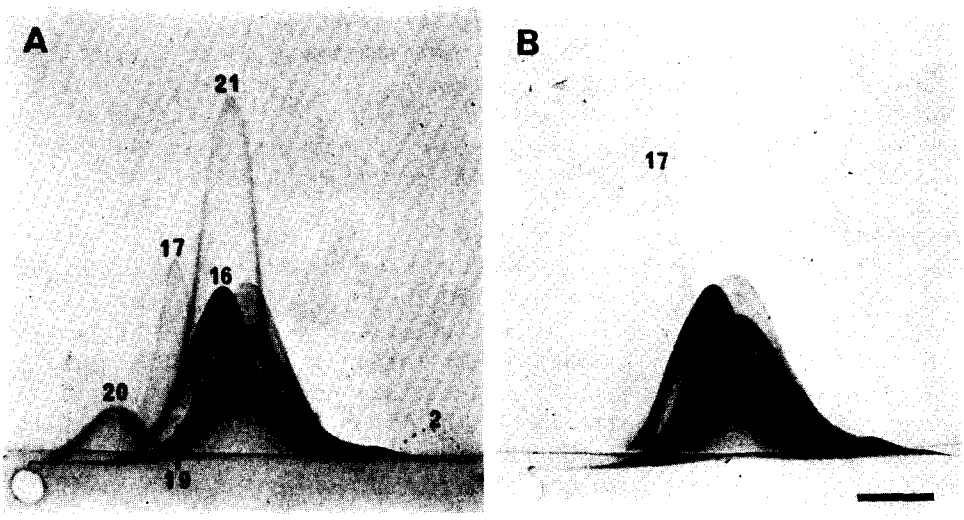

Fig. 1. Crossed immunoelectrophoresis of Berol EMU-043-solubilized human erythrocyte membrane material utilizing A, Normal antibody solution; B, an antibody solution that has been depleted of some antibodies by absorption with intact erythrocytes. Precipitates 2, 19, 20 and 21 are absent in B and the area below precipitate 17 is increased. Precipitate 20 was revealed by esterase staining. In each sample well 22 μg of membrane protein was applied in a volume of 10 μl and electrophoresis was performed in the first dimension for 60 min at 10 V cm^{-1}. The electrophoresis in the second dimension in the antibody-containing gel was performed at 2 V cm^{-1} for 18 h. The amount of antibodies was 0.13 mg per cm^2. 1 % (w/v) Berol EMU-043 was present in all the gels. The gels were stained for esterase with 5-chloro-*o*-toluidine and 1-naphthylacetate and for protein with Coomassie Brilliant Blue. The bar represents 1 cm.

antibodies against antigenic determinants exposed on the outside of the cells, precipitates 2, 19, 20 and 21 did not appear in the crossed immunoelectrophoresis (Fig. 1B). The area below precipitate 17 became larger than in the experiment in Fig. 1A, where a non-depleted antibody solution with about the same total antibody concentration as in Fig. 1B was used, indicating a decrease in the amount of the corresponding antibody in the gel. However, as compared to the other precipitates the area under precipitate 17 increased with the extent of lysis of the erythrocytes during the absorption of the antibodies. An increase was not observed in the experiments where no hemolysis occurred. Precipitate 2 corresponds to serum albumin [1]. No other serum protein is represented among the precipitates [1]. Traces of albumin contaminating the erythrocytes might have precipitated the anti-albumin during the absorption procedure.

The results were independent of the ABO and Rh type of erythrocytes used for the absorption. Precipitates 5-14, 18 (not numbered in Fig. 1; see [1]) and 16 were unaffected by the absorption even when the antibody solution had been absorbed twice with intact erythrocytes (whereas Nos 3, 4 and possibly 15 in [1] were absent).

Antibody solution depleted with isolated membranes

In crossed immunoelectrophoresis with an antibody solution treated with washed, isolated membranes only one precipitate appeared, namely that corresponding

to serum albumin* (not illustrated). This precipitate appeared even when the membranes used in the absorption procedure were from the same preparation as those used in the immunoelectrophoresis and was independent of the ratio between the amounts of membranes and antibodies used in the absorption step. Immunoelectrophoresis gels lacking detergent showed no additional precipitates.

Conclusions

Since the antibodies responsible for precipitates 19–21 were absorbed by the intact erythrocytes the corresponding antigens are at least partially exposed on the outside of the erythrocyte membrane. The behavior of precipitate 17 upon lysis, when the area increased more than the areas below the other precipitates may indicate either that the corresponding antigen is present in the liberated cell content or that the antigen is imbedded in the native membrane becomes exposed upon lysis, for example in the fracture surfaces of the membranes. At the inner membrane surface the antigens corresponding to precipitates 5–14, 16 and 18 are exposed.

The precipitate corresponding to the major glycoprotein

The major glycoprotein (glycophorin) is known to be exposed on the outside of the erythrocyte membrane [22–26] and should therefore correspond to at least one of the precipitates 17, 19 or 21. (Precipitate 20 corresponds to cholinesterase.) Crossed-line immunoelectrophoresis with the isolated major glycoprotein identified it as precipitate 21, since only that precipitate was elevated and was, moreover, fused with the horizontal line precipitate deriving from the major glycoprotein in the intermediate sample gel (Fig. 2A; cf. the control in Fig. 2B).

Ordinary crossed immunoelectrophoresis in the presence of 1 % Berol EMU-043 of the isolated major glycoprotein (Fig. 3) revealed one main precipitate and at least two minor precipitates (arrows), the latter possibly representing impurities. The horizontal, triplicate continuation of the main precipitation line toward the anode (Fig. 2A) with a minor precipitate above (Fig. 3) might correspond to degradation or association or dissociation products of the major glycoprotein (cf. [32] and [27–31], respectively).

The precipitation pattern of the major glycoprotein changed drastically when Berol EMU-043 was not included in the gels, in the analysis of either the purified glycoprotein or of the detergent-solubilized material. In the absence of detergent, the glycoprotein gave a double precipitation line which consisted of a blurred inner line and a sharp outer one (cf. [33]), and which formed two peaks (not illustrated). Furthermore, the migration velocity of the protein increased relative to that of albumin.

Since part of the major glycoprotein is deeply imbedded in the membrane [34–39] it might possess antigenic determinants on the inside of the cell membrane, or within the lipid bilayer, in addition to those exposed on the outside of the cell. In that case the antibody solution depleted by treatment with intact erythrocytes should contain antibody activity against the isolated glycoprotein. This was deliberately

* The amount of albumin corresponded to 0.2 % (w/w) of the membrane protein after the normal washing procedure [1], as determined from the area outlined by the albumin precipitate in crossed immunoelectrophoresis. Another three washings (seven in all) reduced the amount to 0.02 %.

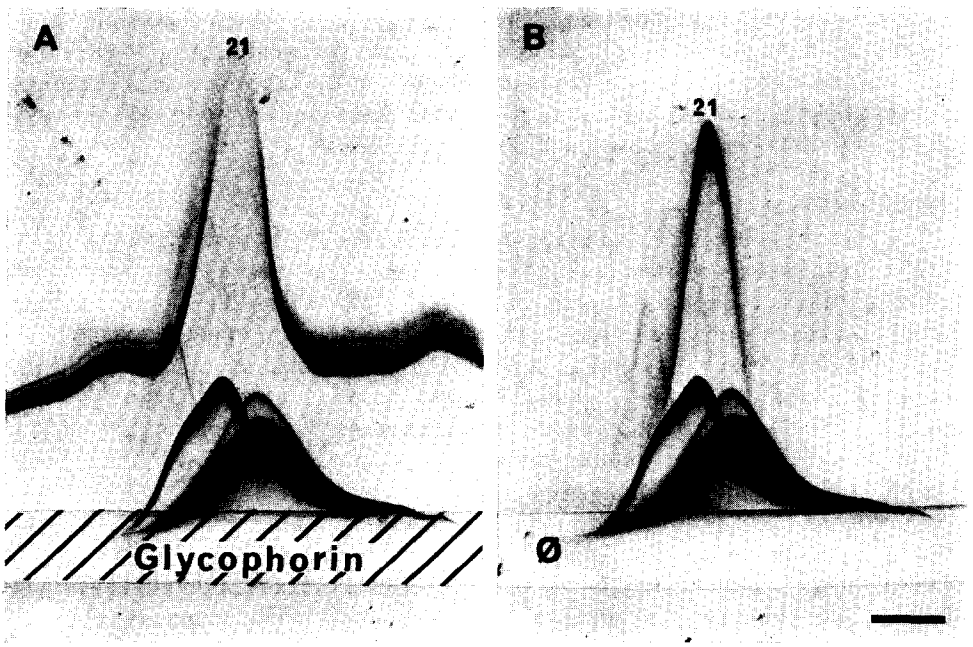

Fig. 2. Identification of the precipitate that corresponds to the major glycoprotein (glycophorin). A, crossed-line immunoelectrophoresis of Berol EMU-043-solubilized human erythrocyte membrane material with 24 μg of the major glycoprotein uniformly incorporated in the intermediate sample gel (the hatched area); B, control with onlybuffer in the intermediate sample gel ϕ. The horizontal line from the major glycoprotein fused with the elevated precipitate No. 21, which indicates identity. In each well 22 μg of solubilized membrane protein was applied in a volume of 10 μl. The conditions were the same as in Fig. 1 except that the electrophoresis time was 70 min in the first dimension.

tested by the sensitive intermediate gel technique [15]. However, crossed immunoelectrophoresis of the purified major glycoprotein or of Berol-EMU-043-solubilized membrane material with the depleted antibody solution in an intermediate gel showed the same patterns for the glycoprotein and for the cholinesterase as controls done with a blank intermediate gel. The same was the case when Berol EMU-043 was omitted from the immunoelectrophoresis gels and from the sample of purified glycoprotein.

DISCUSSION

The principle of the immunoabsorption technique for localization of membrane proteins has been extensively discussed by Johansson and Hjertén in a study of *A. laidlawii* membranes [3].

Immunoabsorption of membrane-specific antibodies with intact erythrocytes has not been detected earlier despite several attempts using double immunodiffusion [40] and immunoelectrophoresis following the method of Grabar [41]. The positive results obtained here are probably accountable to (1) the use of membranes solubilized by a mild procedure, (2) the higher sensitivity of crossed immunoelectrophoresis

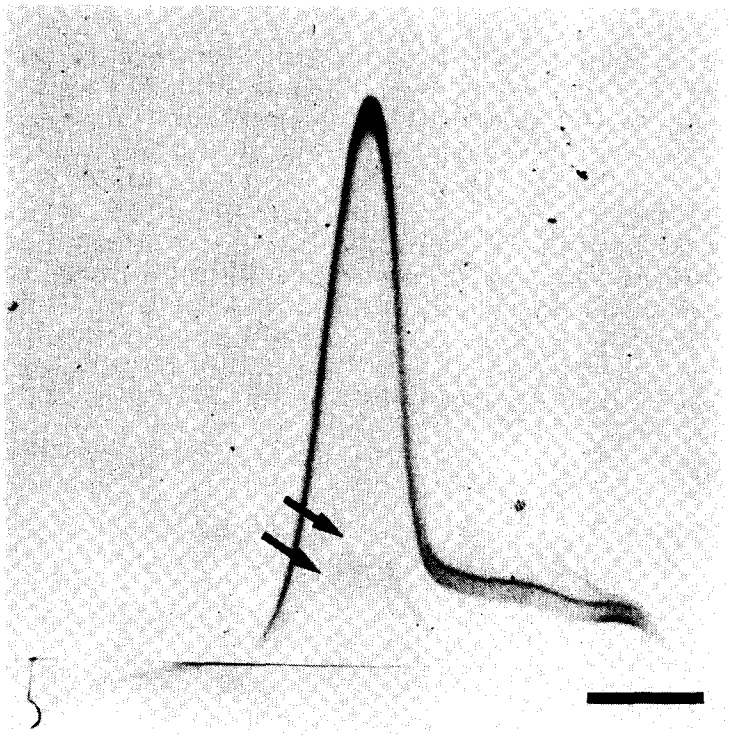

Fig. 3. Crossed immunoelectrophoresis of the partially purified major glycoprotein. Two weak lines are indicated by arrows. The precipitate shows an anodically extended foot below a weak precipitate. A 3-μl-sample of the preparation of the major glycoprotein containing 6 μg of protein was analyzed with 0.16 mg of antibodies per cm^2 in the second dimension. Otherwise the conditions are as indicated in Fig. 1.

[42] as compared to the Grabar technique, and (3) the production and use of immune sera that precipitate most of the solubilized membrane proteins [1].

The acetylcholinesterase of the human erythrocyte membrane is exposed at the outer membrane surface, since the activity of this enzyme is destroyed by proteolytic enzymes outside the intact cell [43]. Proteolytic digestion and chemical labelling studies have shown that the major glycoprotein is also partly exposed at the outer cell surface [22–25], but is at least partly imbedded in [34, 35] or even penetrates the membrane [36–39], although it is relatively resistent to chemical modification from the inside [39, 44]. In the present work antigenic determinants of cholinesterase and of the major glycoprotein are shown to be exposed on the outside of the erythrocyte membrane, in agreement with the already established localization of these proteins. However, we detected no antigenic determinants either of the cholinesterase or of the major glycoprotein either within or on the inside of the membrane. The enzyme might be superficially located, whereas in the case of the major glycoprotein it seems that the imbedded portions of the molecule either are not antigenic or lose antigenicity upon solubilization. A possible shielding effect on antigenic determinants by Berol EMU-

043 can be excluded since the same result was obtained when detergent was omitted from the samples and gels.

Another protein known to be accessible at the exterior surface of the membrane is a protein (numbered 3 by Fairbanks et al. [12]) with a molecular weight of about 100 000 and which spans the membrane [45]. Furthermore, at least five other glycoproteins have been reported to be located on the outer side of the membrane [46]. We do not yet know which of these proteins corresponds to precipitate 19.

The absorption experiments with isolated membranes indicate that all antigenic determinants of the Berol-EMU-043-solubilized membrane proteins are exposed in the lysed membrane, a fact which hardly can be explained only by the exposure of membrane proteins along the surfaces where the disruption takes place. In this connection albumin represents an exception. This protein is possibly absorbed to the membrane during the lysis [47] and seems to be oriented in or on the membrane such that most of its antigenic determinants are not available for the antibodies.

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