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HAS IMMUNOBLOTTING REPLACED ELECTROIMMUNOPRECIPITATION?

EXAMPLES FROM THE ANALYSIS OF AUTOANTIGENS AND TRANSGLUTAMINASE-INDUCED POLYMERS OF THE HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY

The virtues and drawbacks of immunoblotting and electroimmunoprecipitation in the characterization of macromolecules in crude mixtures are presented. Interactions between autoantibodies and human erythrocyte membrane proteins were studied by means of crossed-affinoimmunoelectrophoresis with autologous immunoglobulins incorporated into the first dimension gel and by immunoblotting of sodium dodecyl sulphate-polyacrylamide gel electrophoresis separated erythrocyte membrane proteins with autologous immunoglobulins as primary antibodies. Substrates for transglutaminase in calcium-activated human erythrocyte membranes were examined by immunoelectrophoretic and immunoblotting methods. The experiments concerning autoantibodies complemented each other and showed that epitopes on Band 3 protein, spectrin and ankyrin are recognized by circulating immunoglobulin autoantibodies in normal individuals. The polymer experiments showed the presence of spectrin, ankyrin, Band 3, Band 4.1, glucose transporter, actin and haemoglobin epitopes in the polymer (M_r $3 \cdot 10^6$–$5 \cdot 10^6$). It is concluded that the two techniques complement each other. The most evident advantage of immunoblotting is its sensitivity and applicability while electroimmunoprecipitation in some instances allows an easier identification of distinct protein species and still has a rôle for quantification and certain monitoring purposes.

INTRODUCTION

In the immunochemical analysis of proteins the replacement of immunodiffusion procedures[1,2] with the rocket[3,4] and crossed-immunoelectrophoretic[5,6] techniques represented a significant step forward. Their appearance introduced the era of immunoelectrophoretic procedures, where electrophoresis and detection based on

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precipitation in agarose are the common denominators. Through numerous modifications and combinations of the basic procedures, techniques like the fused-rocket immunoelectrophoresis and tandem and intermediate-gel crossed-immunoelectrophoresis, specific monitoring of chromatographic procedures, characterization of crude mixtures of macromolecules in solution and quantification of both antigens and antibodies can be accomplished[7]. The reactivity and estimation of affinity constants of non-precipitating antibodies and other ligands can be achieved with the crossed-affinoimmunoelectrophoretic procedures[8,9], and it is also possible to characterize microheterogeneity of glycoproteins[7,10] and to identify amphiphilic molecules[7,11,12]. Combinations of a second-dimension immunoelectrophoresis with separations by isoelectric focusing or sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) have also been used[15,16]. The antigen characteristics that can be determined by such means are summarized in Table I.

As a solid-phase hybrid between electroimmunoprecipitation and enzyme-linked immunosorbent techniques, the immunoblotting procedure[34] combines the high resolving powers of different analytical separation methods with the specificity of immunodetection. As for the immunoelectrophoretic techniques, many modifications of the basic principle have emerged[14,19,35–37]. Blotting can be performed in many types of separations and in principle all ligand–acceptor systems can be utilized for visualization. The technique allows a very sensitive characterization of molecules in

TABLE I

FEASIBILITY OF IMMUNOBLOTTING *VERSUS* CROSSED-IMMUNOELECTROPHORETIC METHODS FOR DETERMINING VARIOUS SELECTED CHARACTERISTICS OF ANTIGENS IN CRUDE MIXTURES

Abbreviations: IB = immunoblotting; CIE = crossed-immunoelectrophoresis; t.s. = this study; + = feasible; – = not feasible; (+) = feasible in special cases.

Feature	*Method*		*References*	
	IB	*CIE*	*IB*	*CIE*
Amino acid sequence	+	–	13	–
Size, quaternary structure	+	(+)	14	15
Degradation	+	+	14	17
Charge, p*I*	+	+	18	7
Glycosylation (microheterogeneity)	–	+	19	7
Glycolipids	+	(+)	20	21
Enzymatic activity	(+)	+	22	7
Amphiphilicity	(+)	+	23	7
Membrane topography	+	+	24	25
Protein–protein interaction	+	(+)	t.s.	t.s.
Ligand binding (receptor, transporter)	+	–	26	27
Substrate identification	+	–	28	–
Cell binding	+	–	19	–
Complex formation	+	+	t.s.	t.s.
Derivatization	+	–	29	–
Epitope type (denaturation)	+	(+)	30	31
Epitope mapping	+	–	32	–
Quantification	(+)	+	33	7

complex mixtures[14]. It is useful for antibody characterization, irrespective of the precipitating abilities of the antibody, and it has also been employed in micro-preparative work[38,39]. However, the technique is not yet optimally suited for quantifying purposes. The relative advantages of the two techniques in the determination of selected protein characteristics are listed in Table I.

As a model system, the study of the relationship between ageing phenomena and human erythrocyte membrane proteins illustrates the advantages and drawbacks of these two approaches to the immunochemical characterization of crude antigen preparations. Thus, autoantigenic determinants on the erythrocyte membrane[40] and on the membrane polymer, formed in response to an increase in intracellular Ca^{2+} (refs. 41 and 42), are characterized.

MATERIALS AND METHODS

Proteins and reagents

Human albumin, human transferrin and Ionophore A23187 were obtained from Calbiochem. Behring Corp. (San Diego, CA, U.S.A.), Triton X-100 [polyoxyethylene (9-10) *p-tert.*-octylphenol] scintillation grade from BDH (Poole, Dorset, U.K.), Lubrol PX [polyoxyethylene (10) palmitoyl-stearoyl ester], iodoacetamide, dithiothreitol, phenylmethylsulphonyl fluoride, 3-amino-9-ethylcarbazole, 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue tetrazolium from Sigma (St. Louis, MO, U.S.A.), Amidoblack, Pyronin G and Tween 20 (polyoxyethylene sorbitan ester) from Merck, (Darmstadt, F.R.G.), SDS from Aldrich-Chemie (Steinheim, F.R.G.), aprotinin from Bayer (Leverkusen, F.R.G.), acrylamide and bisacrylamide from Bio-Rad (Richmond, CA, U.S.A.), agarose from FMC Bio Products (Rockland, ME, U.S.A.) (Type LF) and Litex (Copenhagen, Denmark) (Types HSA and HSB). Pepstatin was obtained through courtesy of the United States/Japan Cooperative Cancer Research Program. Stock solutions of the ionophore (5 m*M*) and pepstatin (200 m*M*) were prepared in dimethyl sulphoxide. Nitrocellulose sheets (HAWP, 304 Fo, 0.45 μm) were from Millipore (Bedford, MA, U.S.A.); pore size 0.22 μm was from Schleicher & Schuell (Dassel, F.R.G.); Sepharose 4B and protein A–Sepharose CL4B were from Pharmacia (Uppsala, Sweden) and divinyl sulphone-activated agarose (Minileak medium) was from Kem-En-Tec (Copenhagen, Denmark).

Erythrocyte membranes and lysate

Human erythrocytes were prepared as described[40,42] in a buffer containing 100 m*M* KCl, 60 m*M* NaCl, 10 m*M* glucose and 5 m*M* Tris–HCl (pH 7.4). For the calcium-loading experiments, the cells were suspended at a haematocrit value of 20% and incubated with Ionophore A 23187 at a final concentration of 20 μM. After 15 min, either $CaCl_2$ or $MgCl_2$ was added to a final concentration of 2 m*M*. The cell suspension was then incubated at 37°C for 3 h. Red blood cells were pretreated with 1 m*M* pepstatin by adding the inhibitor to the suspended cells 30 min before addition of ionophore. After addition of Na_2EDTA to a final concentration of 5 m*M*, the cells were washed in the above-mentioned buffer without Ca^{2+}. This wash and the following step were performed at 5°C. Lysis of the cells was performed 20 min after the addition of EDTA by diluting 10 times in 5 m*M* phosphate buffer pH 8, containing 0.5 m*M* phenylmethylsulphonyl fluoride and 1 m*M* iodoacetamide. Membranes were

then isolated according to Dodge *et al.*[43] and were kept frozen at −20°C at a protein concentration of 5 mg/ml, as determined spectrophotometrically at 280 nm[42].

Isolation of polymers

High-molecular-weight material from calcium-loaded erythrocytes, stripped for S–S linked or non-covalently bound proteins, was isolated by means of sucrose gradient centrifugation, as previously described[42]. Isolation of polymers was also performed by chromatography on Sepharose 4B. A 1-mg amount of membrane protein was solubilized in 2% (w/v) SDS in the presence of 2.5 m*M* EDTA and 40 m*M* dithiothreitol and separated at 4°C on a 100 cm × 0.9 cm column (volume, 78 ml) equilibrated with 0.01 *M* potassium phosphate buffer (pH 7.4), 0.1% (w/v) SDS and 1 m*M* EDTA. Fractions of 1.4 ml were collected every 20 min.

Antibodies

The immunoglobulin (IgG) fraction of human plasma was obtained by means of Protein A chromatography followed by dialysis against electrophoresis buffer, as described in ref. 40. The final concentration of IgG was 5–10 mg/ml.

Swine antibodies against rabbit immunoglobulin, peroxidase-conjugated (P 217) or alkaline-phosphatase-conjugated (D 306), rabbit antibodies against human albumin (A 119), human transferrin (A 061), human haemoglobin (A 118) and human erythrocyte membranes (A 104) and alkaline phosphatase-conjugated rabbit antibodies against human IgG (D 336) were obtained from Dakopatts (Copenhagen, Denmark).

The following rabbit antisera against individual erythrocyte membrane proteins were generous gifts: anti-glycophorin [En(a-)absorbed] from C. G. Gahmberg, Helsinki University; anti-band 4.1 from S. E. Lux, Children's Hospital Medical Center, Boston; anti-actin (Band 5) from R. Hynes, Massachusetts Institute of Technology; anti-glucose transporter (Band 4.5) from M. Kasahara and P. Hinkle, Cornell University and anti-band (4.1 + 4.2) from V. T. Marchesi, Yale University. The anti-human haemoglobin antibody was absorbed with completely white ghosts, prepared according to ref. 44. The antibody solution (0.1 ml) was absorbed with 12 mg ghost protein [suspended in 1.32 ml 0.66% (w/v) SDS] overnight at 4°C, followed by centrifugation (35 000 g_{av} for 1 h). The treatment with SDS exposed hidden epitopes of the native proteins, which otherwise were recognized by the unabsorbed antibody on the blot[14]. This incubation time and centrifugation were used for the subsequent absorptions as well.

Immunoabsorption with isolated human erythrocyte membrane polymer (150 μg/ml) was performed by adding 0.6 ml and 0.2 ml polymer per 0.1 ml of the anti-ghost antibody (A104) and the anti-band (4.1 + 4.2) antiserum, respectively.

The antibodies described below were raised in rabbits. For each antigen at least two animals were immunized, using the immunization procedure and schedule recommended by Harboe and Ingild[45]. The early bleedings (6–12 weeks) were found to be most suitable for immunoblotting, whereas the later bleedings were the best for immunoprecipitation. The immunoglobulin fraction (about one-third of the serum volume) was dissolved in 100 m*M* NaCl, containing 15 m*M* NaN_3, with 300 kallikrein units of aprotinin added per ml.

Anti-spectrin antibodies (Anti-band 1 and Anti-band 2). Immunoprecipitates

were used as immunogens[46]. These were cut out from the agarose gels of a line-immunoelectrophoresis of EDTA-extractable human erythrocyte membrane proteins[47], using a polyspecific rabbit anti-ghost antibody. During the electro-immunoprecipitation, the spectrins were partially degraded by plasmin present in the antibody preparation[48]. The specificity of the antibodies was ensured by absorption with spectrin-depleted erythrocyte membranes, which had been repeatedly washed with phosphate buffer, pH 11 and with 6 *M* urea[12,49]. Finally, it was found necessary to absorb with small amounts of washed normal ghosts too small to cause an appreciable reduction in the anti-spectrin titre.

Anti-ankyrin antibody (Anti-band 2.1). The ankyrin-specific immunoprecipitate was excised from a crossed-immunoelectrophoresis of human erythrocyte membrane proteins, solubilized and separated in the presence of 1% (w/v) Berol EMU-043 in Tris–glycine buffer, (pH 8.7)[12]. Under these conditions, the ankyrin precipitate appears in the gel free from other precipitates[12]. Before excision the plates were washed in 100 m*M* NaCl for 20 min and pressed with filter-paper three times. Each rabbit was immunized with the material, obtained from two crossed-immuno-electrophoresis plates, loaded with 40 μg of protein. It was not necessary to absorb this antiserum.

Anti-glycophorin antibody. This was raised against a glycophorin preparation, obtained by the procedure of Marchesi and Andrews[50]. The specificity of the antibody was assured by absorption with EDTA-extracted erythrocyte membrane proteins. Finally absorption with a small amount of washed ghosts was performed, as mentioned for spectrin.

Anti-band 3 protein antibody. That was obtained by immunization with the proteins eluted from the Band 3 region, following SDS-PAGE of erythrocyte ghosts[51]. The purified antibody, which had an equally high titre against Band 3 protein and glycophorin[15], was sequentially absorbed with intact erythrocytes (1 ml compacted erythrocytes per ml antibody solution[48]), a small amount of purified glycophorin and finally with EDTA-extractable membrane proteins[47]. For immunoblotting the two last steps were replaced by absorption with erythrocyte membrane proteins extractable with 6 *M* urea[49]. Before the absorption took place, the membrane proteins were bound to a sheet of nitrocellulose.

Electrophoretic methods

SDS-PAGE was carried out in 1.5 mm thick slab gels (18 cm × 16 cm, Protean apparatus, Bio-Rad, Richmond, CA, U.S.A.). Two gel systems were employed: 5% (w/v) gels with 2.6% cross-linking (bis), prepared according to the method of Steck and Yu[52], and a composite polyacrylamide–agarose gel, consisting of 2% (w/v) polyacrylamide with 5% cross-linking (bis) and 0.5% (w/v) agarose (Type HSB). This gel was cast according to the methods of Peacock and Dingman[53] with 0.2% (w/v) SDS, using the buffer and catalyst formulation given by Steck[54]. The lower 5 cm of the gel consisted of 2% (w/v) HSB agarose. Application slits were made with a specially designed comb, forming six slits 0.8 cm wide. Each tooth could be removed separately to avoid cracks in the gel because of the low gel strength. Gels overlaid with electrophoresis buffer were left to stand overnight before use. Samples (15 μl) of membrane proteins (125 μg) were applied after solubilization in 2% SDS and 40 m*M* dithiothreitol (15 min, 56°C). Electrophoresis at a controlled buffer temperature of

12°C was performed, applying 40 mA per gel side. The electrophoresis was terminated when the tracking dye pyronin had migrated 7.2 cm.

Definitions and procedures for fused-rocket, line and crossed-immunoelectrophoresis, crossed-immunoelectrophoresis with an intermediate gel and crossed-immunoelectrophoresis with interaction in first dimension electrophoresis are given by Bjerrum and Bøg-Hansen[46]. The 1% (w/v) agarose gels contained 0.1 or 0.5% (v/v) of Triton X-100. Solubilization of erythrocyte membrane to a protein concentration of 2 mg/ml was performed as described in ref. 12. Staining for esterase was performed according to ref. 55.

A combination of SDS polyacrylamide (5%, w/v) and agarose (1%, w/v) crossed-immunoelectrophoresis was carried out as described by Bjerrum *et al.*[12,16]. SDS-PAGE was performed as described above. After electrophoresis, the polyacrylamide slab was trimmed to a width of 0.75 cm and was washed for 15 min at 22°C in a buffer containing 38 m*M* Tris, 100 m*M* glycine (pH 8.7) and 1% (w/v) Lubrol PX. The slice was then placed 2 cm above the cathodic edge of the 10 cm × 7 cm plate used for crossed-immunoelectrophoresis, on top of a 2 mm thick and 4 cm wide agarose layer, which contained 3.5% (w/v) of Lubrol PX. The two layers were then sealed by application of a few drops of warm agarose (with 2%, w/v, Lubrol PX) at the cathodic side. The antibody-containing agarose with 2% (w/v) Lubrol PX was cast as a 6 cm wide and 1.5 mm thick layer on the anodic side of the plate. To spare costly antibodies, they were applied in smaller "windows", corresponding to the expected appearance of the corresponding antigens of the SDS gel. Electrophoresis was carried out at 2 V/cm for 16 h, using the Tris–glycine buffer of pH 8.7. The polyacrylamide gel was removed prior to pressing, washing and staining of the agarose.

For immunoblotting, the procedure originally described by Towbin *et al.*[34] was followed, using the modifications of Heegaard and Bjerrum[56]. For the polymer analysis, electrotransfer to nitrocellulose in a buffer tank was performed[56]. The positions of the lanes on the nitrocellulose sheet were indicated by the dye Pyronin G, which was added to the slits at the start and the end of the electrophoresis. The amount of protein transferred was visualized by Amidoblack staining[56].

Blocking was performed at room temperature by incubation in 40 ml of washing buffer (50 m*M* Tris–HCl, 150 m*M* NaCl and 5 m*M* NaN_3, pH 10.2), containing 2% (v/v) Tween 20 for 2 min giving rise to a completely white background[56]. After washing with peroxidase staining buffer (50 m*M* acetate buffer, pH 5.5), incubation with the peroxidase-conjugated swine anti-rabbit antibody preparation (1 μl/ml) took place for 2 h with gentle shaking. Aminocarbazole was used for peroxidase staining[56].

For the autoantigen experiments, the semi-dry electroblotting procedure was employed[40]. After electrotransfer, the blots were washed and probed with different concentrations of the Protein A-purified autologous immunoglobulin fractions (5–10 mg/ml) or rabbit anti-erythrocyte membrane protein antibodies diluted (1:2000) in washing buffer (pH 10.2)[56]. Colloidal gold was used for general protein staining[56]. Bound human or rabbit IgG was visualized after incubations with alkaline phosphatase-conjugated rabbit anti-human IgG or swine anti-rabbit immunoglobulin antibodies, respectively using Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the substrate[40].

RESULTS

Identification of autoantigens

Crossed immunoelectrophoresis was employed for the study of possible autoantibodies directed against erythrocyte membrane proteins, which might be responsible for the removal of aged erythrocytes[40]. With a polyspecific rabbit anti-ghost antibody preparation, the reference precipitation pattern of erythrocyte membrane proteins, solubilized in non-ionic detergent, shown in Fig. 1A, was established[12]. A modification of the technique, where a free ligand is present in the first-dimension electrophoresis, can reveal interactions with high sensitivity[9,57]. Binding is revealed as migrational differences of the interacting protein(s), measured as the position of the precipitate in question relative to an internal marker protein. The marker in this case is albumin, which has been added to the membrane preparation and which is precipitated by anti-albumin antibodies present in the anti-erythrocyte membrane antibody preparation.

Fig. 1 shows that incorporation of Protein A-purified autologous immunoglobulin in increasing amounts in the first dimension selectively affects the migration of the cytoskeletal proteins ankyrin (2.1), spectrin (1, 2) and the integral membrane protein band 3[7,10,57]. The shifts in mobility are likely to be due to binding of almost non-migrating immunoglobulin molecules in the first dimension, while glycophorin

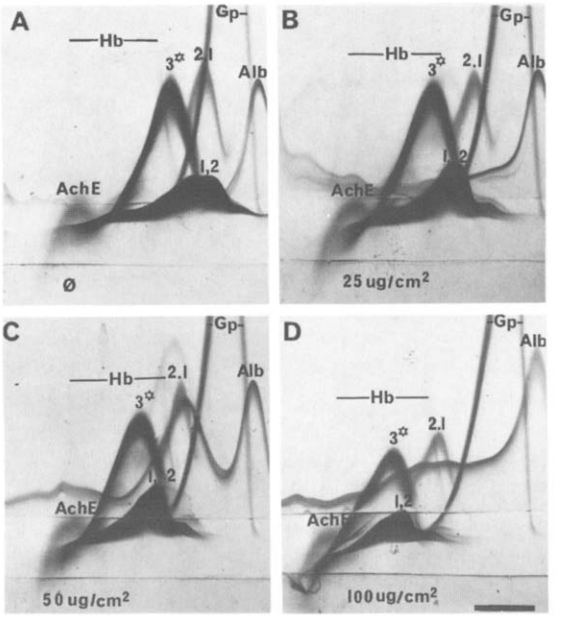

Fig. 1. Crossed-immunoelectrophoresis of Triton X-100-solubilized human erythrocyte membrane proteins with increasing amounts of autologous Protein A-purified immunoglobulin fraction of serum in a volume of 1 ml, incorporated into the first-dimension gels of B, C and D. A is the reference. The anti-ghost antibody content of the second dimension gel was 6 μl/cm^2. The following precipitates are indicated: 1 and 2 (spectrin), 2.1 (ankyrin), 3* (Band 3 protein complex), Gp (glycophorin), Alb (albumin), AchE (acetylcholinesterase, separately stained[52]), and IIb (haemoglobin)[12]. The albumin precipitate shows various degrees of tailing due to the presence of albumin in the Protein A-eluate. Bar = 1 cm.

(Gp), acetylcholinesterase (AchE) and haemoglobin (Hb) are unaffected.

A comparable activity was present in an autologous IgG preparation, purified by means of immunosorbent chromatography of the Protein A eluate on columns with immobilized rabbit anti-human IgG[40].

The presence of autoantibodies can also be studied by means of immunoblotting procedures[19,33,35,36,58]. Fig. 2 shows the outcome of an immunoblot of SDS-solubilized and dithiothreitol-reduced erythrocyte membrane proteins subjected to SDS-PAGE and incubation with autologous immunoglobulin after electroblotting. Visualization of IgG binding was performed by means of alkaline-phosphatase-labelled anti-human IgG antibodies. Reactivity with most of the protein bands present on the blot is apparent (Fig. 2, lane b). This binding ability was not influenced by absorption of the autoantibody preparation with intact erythrocytes, while immunoabsorption with crude erythrocyte membrane preparations removed the activity (Fig. 2, lane c). Thus the anti-human IgG alkaline conjugate did not react with the erythrocyte proteins. These experiments indicate that the majority of epitopes recognized by the circulating autoantibodies are situated inside the erythrocyte, *i.e.*, on cytoskeletal proteins and on intracellulary parts of the integral membrane protein band 3.

In conclusion, these experiments illustrate the value of the crossed-immunoelectrophoretic technique, where a combination of precipitate morphology, migration and immunoreactivity gives information in addition to the high resolution and sensitivity of the immunoblotting procedure itself.

Isolation of polymer materials generated in erythrocytes

Erythrocytes contain an γ-glutamyl-ε-lysine transglutaminase[41], which upon activation with Ca^{2+} cross-links the membrane proteins. The physiological rôle of this enzyme is not known, although it has been suspected to be involved in the removal of

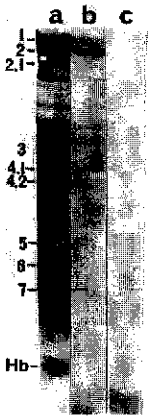

Fig.2. Immunoblots of human erythrocyte membrane proteins (30 μg in each lane), separated by SDS-PAGE (anode at the bottom) and electroblotted on nitrocellulose. (a) Gold-stained transfer; (b) incubation with 1:180 dilution of Protein A-purified autologous immunoglobulin fraction; (c) as in (b) but absorbed with crude erythrocyte ghosts. Visualization by means of an alkaline-phosphatase-conjugated rabbit anti-human IgG antibody, followed by staining with Nitroblue tetrazolium with 5-bromo-4-chloro-3-indolyl phosphate as a substrate[56]. Band assignments according to Steck and Yu[53].

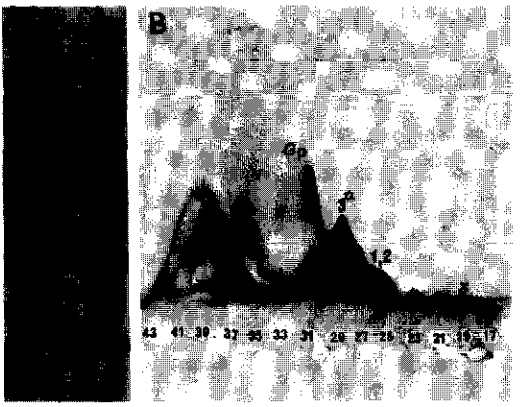

Fig. 3. (A) Effect of Ca^{2+} on the membrane profile in intact human erythrocytes. Lanes: a, SDS-PAGE pattern of membrane material from cells exposed to 2 m*M* Ca^{2+} for 3 h at 37°C at 20% haematocrit with 20 μM ionophore; b, corresponding control incubated with 2 m*M* Mg^{2+}. Note the disappearances of Band 2.1 and Band 4.1 and the production of high-molecular-weight membrane protein polymer (X). (B) Fused-rocket immunoelectrophoresis in the presence of 2% Lubrol PX of an SDS-solubilized membrane material from calcium-incubated cells, separated by means of Sepharose CL4B chromatography. The immunoprecipitated material in fractions 17–20 represents high-molecular-weight material, corresponding to X of lane a in (A). The anti-ghost antibody content of the gel was 6 $\mu l/cm^2$. To avoid interference from the SDS in the samples, 5 μl 20% (w/v) Lubrol PX was added to each well before electrophoresis. Designations correspond to those of Fig. 2.

aged erythrocytes[41,59,60]. However, the intracellular free calcium levels occurring physiologically are by far lower than those employed in the present study.

The polymer is generated upon incubation with Ca^{2+} in the presence of the Ionophore A23187 and is visible on top of the gel in SDS-PAGE (Fig. 3A, X). The polymer can be isolated by gradient ultracentrifugation in the presence of a non-ionic detergent[42] or by gel chromatography. The fused-rocket immunoelectrophoresis profile of the latter experiment is shown in Fig. 3B and illustrates the separation obtained in the presence of 0.1% SDS of the high-molecular-weight polymeric material from the normal membrane proteins. Interference of SDS with the immunoprecipitation was avoided by the presence of the non-ionic detergent in the wells and gel. Only fractions 17–20 were used as the source of the polymer whereby contamination of the spectrin was avoided.

Immunoelectrophoretic epitope analysis of the polymers

With the crossed-immunoelectrophoretic pattern of normal human erythrocyte membrane antigens as a reference, the effect of absorbing the polyspecific anti-ghost antibody with isolated polymers was examined (Fig. 4).

When the absorbed antibody was included in the intermediate gel (as in Fig. 4A) rather than the original antibody (as in Fig. 4B), an upward displacement in the heights of the precipitation lines for spectrin (1 and 2), ankyrin (2.1) and Band 3 proteins (only to a small degree) was observed. This is consistent with the idea that these protein components are present in the polymeric material, as the titre against the rest of the precipitated components including haemoglobin was undisturbed[42].

Since no precipitates corresponding to the Band 4.1 and 4.2 polypeptides were

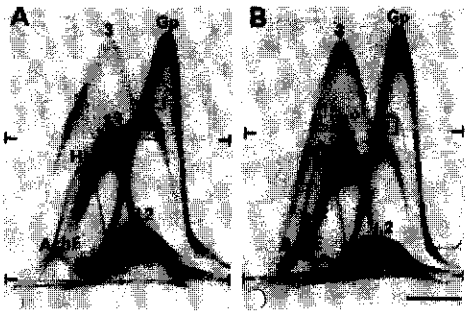

Fig. 4. Crossed-immunoelectrophoresis with an intermediate gel containing anti-human erythrocyte membrane antibody absorbed with isolated membrane polymer. For details of absorption, see Materials and Methods. Normal human erythrocyte ghost (8 μg of protein), solubilized in Triton X-100 at pH 10^{12}, was applied for electrophoresis. The absorbed antibody (3 μl/cm^2) was included in the intermediate gel (between the markings) in (A). The intermediate gels for (B) contained the unabsorbed antibody (3 μl/cm^2). The upper gels in both experiments contained unabsorbed antibody (5 μl/cm^2). Designations correspond to those of Fig. 1.

identified in the precipitation pattern, the presence of such epitopes was investigated in a separate experiment, in which an oligospecific anti-band 4.1 and 4.2 antibody was used. Since the antiserum was raised against material cut from SDS-PAGE gels, it did not react with Triton X-100-solubilized material[15]. The specificity of the Anti-band 4.1 and 4.2 antiserum was therefore first tested by SDS-PAGE crossed-immunoelectrophoresis (Fig. 5). Upon incorporation of the antibody preparation in an intermediate window (Fig. 5A), two immunoprecipitates were observed at the bottom line of the gel. Also, the glycophorin precipitate was slightly affected, as an extension of its legs appeared in the intermediate gel (compare Fig. 5A, B and C). That these two lower precipitates corresponded to Band 4.1 and Band 4.2, respectively, was established as follows. Upon incubation of erythrocytes with Ca^{2+}, Band 4.1 disappeared from the SDS-PAGE pattern[41,60] and so did the cathodic precipitate (Fig. 5B). Furthermore, the positions of the two precipitates strictly correlate with Band 4.1 and Band 4.2, as measured relatively to the markers, human transferrin and albumin (Fig. 5C and D), both of which retain their immunoreactivity after SDS-PAGE.

The Anti-band 4.1 and 4.2 antiserum was absorbed with purified polymer and applied in SDS-PAGE crossed-immunoelectrophoresis of normal erythrocyte membrane proteins (Fig. 6B). By comparison with the control (unabsorbed antiserum) (Fig. 6A) it appeared that the precipitate corresponding to Band 4.1 disappeared, indicating the presence of this protein in the polymer. The precipitate of band 4.2 appeared larger but less strongly stained, and by absorption with a triple amount of polymer material this precipitate also disappeared. This may indicate that the polymer also contains some epitopes of Band 4.2. A similar dilution of the antibody employed by buffer from a blank sucrose gradient did not have this effect.

Immunoblotting analysis of the constituents of the polymer

The immunoblotting technique is very sensitive and the demand for monospecificity of the antibodies employed is equivalently high. It was therefore found necessary to perform extensive absorption of the available antibodies. Fig. 7 shows the

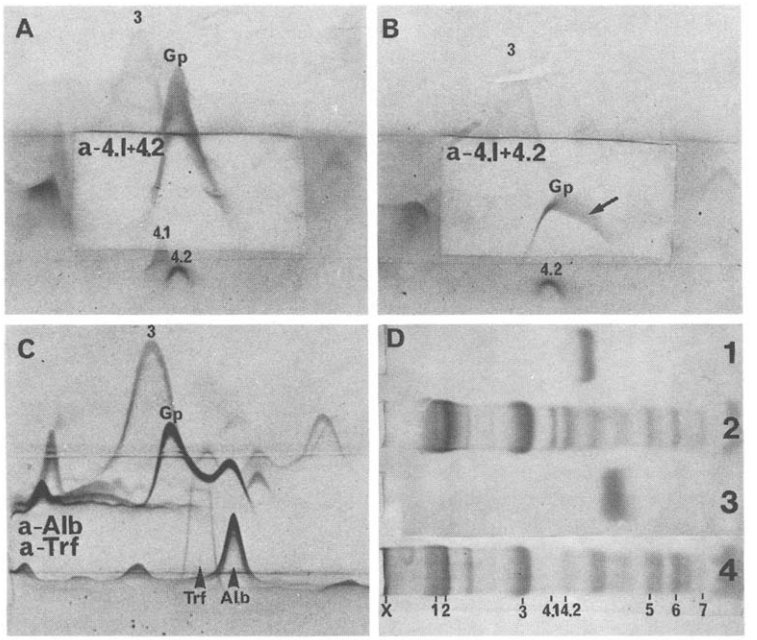

Fig. 5. Identification of Band 4.1 and Band 4.2 precipitates in SDS-PAGE crossed-immunoelectrophoresis. Immunoelectrophoretic analysis of 40 μg membrane proteins from normal erythrocytes (A and C) and of membrane proteins from 3-h calcium-incubated cells (B). Note that glycophorin is degraded (arrow). In (C) the proteins were mixed with 1.5 μg human albumin and 0.5 μg human transferrin prior to electrophoresis. The intermediate gel contains in (A) and (B) Anti-band (4.1 + 4.2) antiserum (20 μl/cm^2), applied in a "window". In (C) it contains 4 μl/cm^2 of anti-human albumin (a-Alb) and 4 μl/cm^2 of anti-human transferrin (a-Trf). The upper gels contain antibodies against normal human erythrocyte membranes: 9 μl/cm^2 in (A) and (B), 15 μl/cm^2 in (C). (D) The resolution obtained by first-dimension SDS-PAGE (anode to the right). The albumin and transferrin markers are shown in lanes 1 and 3, respectively. Erythrocyte membrane proteins from normal cells and from 3-h calcium-loaded cells are shown in lanes 2 and 4, respectively. On incubation with Ca^{2+}, Band 4.1 is missing. Designations and other conditions correspond to those of Fig. 2.

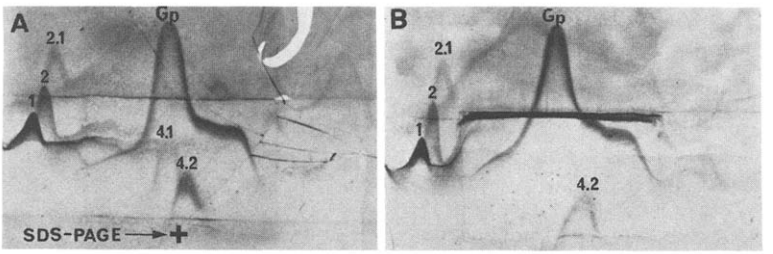

Fig. 6. Analysis for epitopes of Band 4.1 and Band 4.2 on purified polymer. SDS-PAGE crossed-immunoelectrophoresis of normal erythrocyte membrane proteins with an intermediate gel, containing, in (B), 21 μl/cm^2 of Anti-band (4.1 + 4.2), absorbed with 15 μg SDS-isolated polymer, and in (A) the corresponding control with unabsorbed antiserum (21 μl/cm^2). Both antibodies were applied in a "window". Other conditions and designations correspond to those of Fig. 5.

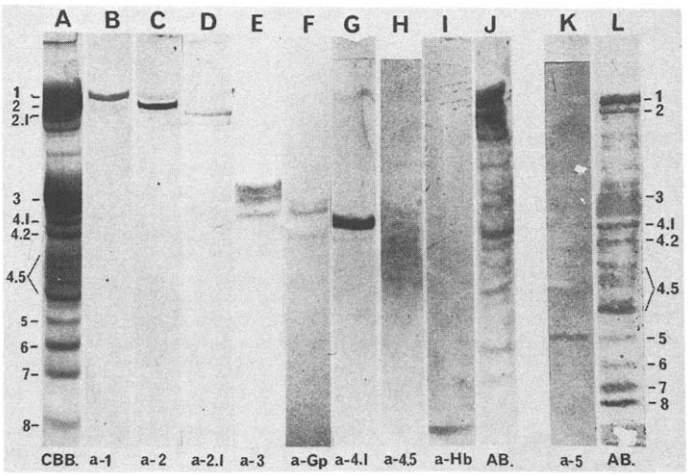

Fig. 7. Antibody specificity, tested by immunoblotting. Human erythrocyte membrane proteins were separated by SDS-PAGE (A–J), according to Steck and Yu[52] and (K, L) according to Laemmli[63]. Anode at the bottom. Lane A shows a Coomassie brilliant blue (CBB)-stained gel (50 μg protein); lanes B–L are nitrocellulose strips with electrophoretically transferred proteins, stained (J, L) with Amidoblack (AB) (50 μg protein) and (B–I, K) for peroxidase activity (10 μg protein) after incubation with monospecific rabbit antibodies, followed by a peroxidase-conjugated swine anti-rabbit immunoglobulin. The primary rabbit antibody was Anti-band 1 (diluted 1 + 666) (B), Anti-band 2 (1 + 333) (C), Anti-band 2.1 (1 + 333) (D), Anti-band 3 (1 + 200) (E), Anti-glycophorin (1 + 500) (from Dr. Gahmberg) (F), Anti-band 4.1 (1 + 2000) (an artefactual staining is seen in the spectrin region) (G), anti-glucose transporter (a-4.5) (1 + 2500) (H), anti-haemoglobin (a-Hb) (1 + 200) (I) and anti-actin (a-5) (1 + 25) (K). Designations as in Fig. 2.

resulting specificity of these antibodies in immunoblotting. Antibodies against Band 1, Band 2, Band 3, haemoglobin and actin give one band in the blots. Where several bands are seen, it can be explained by molecular-weight heterogeneity, as for glycophorin[61], glucose transporter[62] and probably also for Band 3 protein. Proteolytic cleavage may be the reason for the two bands seen with the Anti-band 2.1 antibody[12]. The high-molecular-weight polymeric material can be analysed in the same way, because this material migrates in an SDS-containing composite gel, consisting of 2% acrylamide and 0.5% agarose. The polymer is separated from the rest of the erythrocyte membrane proteins (Fig. 8, lane A) and appeared under these conditions as a symmetrically distributed polydisperse band with a molecular weight between $3 \cdot 10^6$ and $5 \cdot 10^6$ (Fig. 8, X).

The polymeric material can easily be transferred from the electrophoresis gel to the nitrocellulose sheet, as demonstrated by subsequent staining with Amidoblack (Fig. 8, lane L). When the absorbed monospecific antibodies, used in the same concentrations as in Fig. 7, were applied to such transferred proteins, a staining of the polymer band was observed for all antibodies employed, except that directed against glycophorin (Fig. 8, lanes B–J). Two different anti-glycophorin antibody preparations showed consistent results. The reaction of the anti-actin antiserum (lane I) with the polymer was weak, but clearly visible in the freshly stained immunoblots. Furthermore, in the concentration range applied, sera and IgG preparations from non-immunized rabbits did not react with the blotted erythrocyte membrane protein material (Fig. 8, lane K).

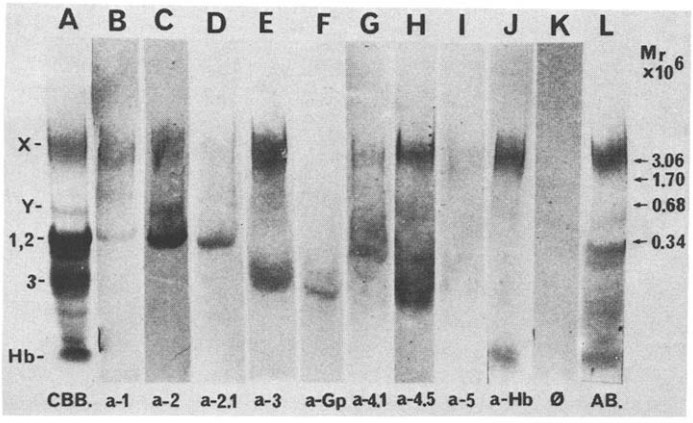

Fig. 8. Demonstration by immunoblotting of the presence of epitopes of individual membrane proteins on the polymeric protein material, generated in calcium-loaded human erythrocytes in the presence of pepstatin. The proteins were separated on SDS-containing composite gels of 2% polyacrylamide and 0.5% agarose. Molecular-weight markers are indicated on the right on the basis of transglutaminase mediated cross-linked fibrin oligomers. Lane A shows the Coomassie brilliant blue-stained gel, loaded with 125 μg protein. The polymeric material appeared as a broad band X in the molecular-weight range of $3 \cdot 10^6$–$5 \cdot 10^6$ well separated from the spectrins, $M_r \approx 2.2 \cdot 10^5$ (1, 2) and as a new, discrete band with $M_r \approx 5 \cdot 10^5$ (Y). Lanes B–K are nitrocellulose strips with electrophoretically transferred proteins (125 μg), stained (K) with Amidoblack and (B–L) for peroxidase activity. The primary monospecific antibodies, used in the same concentrations as in Fig. 7, were applied as stated on the Figure. Lane K served as a control where a non-immune rabbit serum was employed (1 + 1000) (Ø). Other conditions and designations as in Fig. 7.

The specificity of the antibody binding to the polymer was further evidenced by the simultaneous binding to the corresponding individual antigens of lower molecular weight. In this respect, the behaviour of the Anti-band 4.1 antibody was abnormal. Aside from its reaction with the polymer, it also reacted with normal membrane material of higher molecular weight than expected (Fig. 8, lane G). This observation

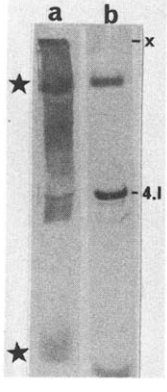

Fig. 9. Demonstration by immunoblotting of the distribution of Band 4.1 epitopes after SDS-PAGE, performed according to Steck and Yu[52], of erythrocyte membrane protein from (a) calcium-loaded cells in the presence of pepstatin and (b) magnesium-loaded cells (control). The primary antibody was Anti-band 4.1 (1 + 2000). Note the continuous distribution of immunoreactivity from the position of Band 4.1 to the top of the gel. The asterisks indicate the transferred track of the marker stain Pyronin G. Conditions and other designations as in Fig. 7.

was verified in a separate immunoblotting experiment, where polymer-containing erythrocyte membrane material was separated by conventional SDS-PAGE (Fig. 9).

Thus, the two immunochemical approaches employed for epitope analyses of the transglutaminase-generated polymers in the erythrocyte membrane complement each other. Both techniques show that spectrin, ankyrin, Band 3 protein and Band 4.1 participate in the polymer formation. Immunoblotting gave further evidence for the incorporation of both spectrins (Band 1 and 2), haemoglobin, glucose transporter (4.5) and actin in the polymer. The crossed-immunoelectrophoresis approach seems not to be sensitive enough to demonstrate a decrease in the titre of antibody against haemoglobin after absorption (*cf.*, Fig. 3) but is capable of showing that Band 4.2 epitopes probably are also involved.

DISCUSSION

The demonstration of circulating IgG autoantibodies against human erythrocyte membrane proteins illustrates how the techniques of crossed-affinoimmunoelectrophoresis and immunoblotting complement each other. By the former technique, interactions with cytoskeletal and integral membrane proteins, excluding glycophorin, were observed. Immunoblotting experiments with absorbed antibodies then showed that the autoantibodies reacted mainly with epitopes on the inside of the cell membrane. Immunoblotting requires only small amounts of reagents and the experiment with absorbed antibodies would have been cumbersome to perform by means of immunoelectrophoretic techniques.

A further advantage of immunoblotting in this connection is that the class and specificity of the interacting molecules are demonstrated directly by the visualization step. The crossed affinoimmunoelectrophoresis, on the other hand, gives a clearer impression of which proteins are involved in the interaction.

Glycophorin, which is also an integral membrane protein and which is easily identifiable in the precipitation pattern, is evidently non-reacting (Fig. 1). Thus, these techniques give results which, taken together, are easier to interpret than the results of either of these techniques alone. In the experiments on polymer formation, glycophorin was further demonstrated not to participate in the cross-linking events. This series of experiments (Figs. 3–9) shows how connoisseur modifications of basic procedures make it possible to extract approximately the same amount of information by both techniques, although immunoblotting in most instances represents the quickest and less difficult approach. Special problems arose when the antibodies precipitated only SDS-treated material (the Anti-band 4.1 and 4.2 antibody preparation) but this was circumvented by means of special modifications of the crossed-immunoelectrophoresis procedure (Fig. 5). Immunoblotting did not demand any special modifications to show the presence of Band 4.1 epitopes on the polymer (Fig. 8), and the presence of Band 4.1 epitopes in a wide range of molecular weights was further demonstrated (Fig. 9). This observation, together with the early disappearance from the SDS pattern after addition of Ca^{2+}, points to the Band 4.1 protein as an important substrate for the enzyme. Unfortunately, the electrophoresis system employed did not separate Band 4.1 in 4.1a and 4.1b. The ratio between these bands differs with age[64]. Immunoblotting requires a very high degree of monospecificity of the primary antibodies, which ideally is accomplished by means of monoclonal antibodies.

Furthermore, immunoelectrophoresis allows direct demonstration of antigens with catalytic activities. Thus, the polymer has been shown to possess catalase activity, whereas acetylcholinesterase activity was shown to be completely independent of the polymer entities[65].

For quantification of antigens and antibodies, immunoelectrophoresis also takes the lead, while the sensitivity, high resolving power, broad applicability, independence of precipitating abilities of the employed antibodies and low consumption of reagents constitute the main advantages of immunoblotting. In addition, studies of interactions by means of immunoblotting techniques can be performed with an high degree of freedom in the selection of ionic strength, pH and other buffer manipulations. This is in contrast to the immunoelectrophoretic techniques, which are much more restricted in this respect. On the other hand, in certain instances the latter techniques will, as illustrated by the experiments with autoantibodies against human erythrocyte membrane protein components, give more information about the identity of the interacting macromolecules than the immunoblots. Also, the positions and other characteristics (shape and stainability) of precipitates can offer additional information for identification which is not given by ordinary immunoblotting procedures as the use of the oligospecific antibody preparation in the experiment shown in Fig. 5 illustrates. Finally, for quantification, including monitoring of column chromatography experiments (*cf.*, fused-rocket immunoelectrophoresis in Fig. 3), and for the study of glycosylation microheterogeneity the electroimmunoprecipitation methods are valuable.

In conclusion, the two immunochemical methods complement each other. For antigen characterization (Table I) the two methods are almost equally suited. However, as immunoblotting normally relies on SDS-PAGE methods, which are part of the standard protein analysis armament of most laboratories, while crossed-immunoelectrophoresis methods often are regarded as troublesome, it may be said that immunoblotting in general is the easier method of the two. As an extension of routine protein analyses, immunochemical characterization by means of immunoblotting is thus the straightforward choice. As mentioned, the flexibility and versatility of the immunoblotting principle is greater than that of the electroimmunoprecipitation techniques. Therefore, the latter will most likely in many instances in the future be replaced by immunoblotting procedures as the almost exponential growth of publications on immunoblotting methods nicely illustrates[36].

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