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Immunochemical characterization of interactions between circulating autologous immunoglobulin G and normal human erythrocyte membrane proteins

Niels H.H. Heegaard

The Blood Bank, Rigshospitalet, Copenhagen and The Protein Laboratory, University of Copenhagen, Copenhagen (Denmark)

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Autologous immunoglobulin G present during electrophoresis of human erythrocyte membrane proteins influenced the electrophoretic mobility of some of the proteins. Different types of non-ionic detergents were used for solubilization of the membranes and together with experiments using dimyristoylphosphatidylcholine-derived erythrocyte membrane vesicles this indicated that IgG binds to spectrin, ankyrin, and band 3 protein. The binding was independent on proteolysis and not due to unspecific protein–protein interactions. Immunoblotting experiments also showed binding to polypeptide bands in the spectrin and ankyrin regions and demonstrated the presence of erythrocyte-associated IgG. The reactivity may be due to natural autoantibodies involved in the clearance of cellular debris in vivo. Whether the observations are of relevance for the putative immune-mediated clearance of old erythrocytes from the circulation remains to be established.

Introduction

The interplay between plasma proteins and the surface of human erythrocytes has been studied for many years [1–3]. It is well established that complement proteins and immunoglobulins are present on red blood cells [4] and that immunoglobulin G (IgG) autoantibodies against epitopes of membrane proteins normally are present in the circulation [5–7].

By means of affinity electrophoresis in agarose gels it is possible to study dynamic aspects of macromolecular interactions. In this way crossed affinity immunoelectrophoresis revealed a selective interaction between protein A-purified autologous immunoglobulin and solubilized human erythrocyte membrane proteins. Immunoglobulins bound to Triton X-100-solubilized forms of the integral membrane protein, band 3 protein in a complex precipitate, and to the peripheral membrane proteins spectrin and ankyrin [8,9].

The involved epitopes, the specificity of the interaction, and the dependence of the interaction on conformation and complex formation are here further examined by means of immunoblotting and affinity immunoelectrophoretic methods using different non-ionic detergents and extraction procedures.

Materials and Methods

Chemicals. Tris 7–9 (tris[hydroxymethyl]aminomethane), phenylmethylsulphonyl fluoride (PMSF), dimyristoylphosphatidylcholine (DMPC), pepstatin A, *N*-ethylmaleimide, dithiothreitol, Lubrol PX (ethylene oxide condensates of fatty alcohols), 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt), Nitroblue tetrazolium and iodoacetamide came from Sigma. Tween 20 (polyoxyethyethylene sorbitan ester), Pyronin G, glycine, silver nitrate, sodium azide, Amidoblack, polyethylene glycol 20000, and EDTA were purchased from Merck. Pharmacia delivered Dextran T70, protein A-Sepharose CL4B and protein G-Sepharose 4 FF. Divinylsulphone-activated agarose (Minileak low), its β-mercaptoethanol derivative (T-gel), and immobilized jacalin were from Kem-En-Tec A/S, Triton X-100 (*iso*-octylphenoxyethoxyethanol) from BDH, agarose, type HSA from Litex, and aprotinin, 20000 kIE/ml from Bayer. Nitrocellulose membranes, BA 83 pore size 0.2 μm were obtained from Schleicher & Schuell. Acrylamide, *N*,*N*′-methylene bisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, and ammonium persulphate were

Correspondence: N.H.H. Heegaard, NIMH Neurosciences Center at Saint Elizabeths, 2700 Martin Luther King, Jr. Ave. SE, Washington, DC 20032, U.S.A.

from Bio-Rad while sodiumdodecyl sulphate (SDS) was purchased from Serva.

Antibodies and proteins. Dakopatts delivered normal rabbit serum (X902, lot 032A) and rabbit antibodies against human serum proteins (A206, lot 013), human erythrocyte membrane proteins (A104, lot 015 and 039), IgG (A090, lot 018) and against IgA (092, lot 018) and alkaline phosphatase-conjugated rabbit antibodies against human IgG (D336, lot 058). Human serum albumin (Reinst) was from Behringwerke AG.

Methods. Blood was obtained from healthy adult donors of various ABO- and Rhesus-types either in adenine-citrate-dextrose or in 5.5 mM EDTA (pH 7.4). Plasma was obtained by centrifugation ($1350 \times g_{av}$ for 10 min) and converted to serum by the addition of 1 ml 1 M $CaCl_2$ to 100 ml plasma. The immunoglobulin fraction of serum was purified by means of protein A- or protein G-Sepharose chromatography. Immunoglobulin G was further purified from the eluates by means of immunosorbent chromatography on a 15 ml Minileak column containing immobilized rabbit anti-human IgG antibodies [8]. The gel had a capacity of about 0.8 mg IgG/ml gel matrix and apart from traces of albumin the eluate as judged by crossed immunoelectrophoresis with anti-serum protein antibodies [10] contained only IgG. Washing buffer for all columns was 0.1 M Tris-HCl, 1 M NaCl, 15 mM NaN_3 (pH 8.6), elution buffer was 0.1 M citrate/HCl (pH 2.5), and neutralizing buffer was 1 M Tris-HCl (pH 9.0). The outcome of the columns was monitored at 280 nm.

IgA was purified from the serum of a myeloma-patient (IgA, kappa light chains) by jacalin affinity chromatography followed by hydrophobic interaction chromatography on a T-gel (Lihme, A.O.F., Frandsen, N.M. and Heegaard, P.M.H., unpublished data). The IgA concentration after purification and dialysis was 3.4 mg/ml. The preparation contained traces of albumin and distinguishable types of normal and pathological IgA species in a crossed immunoelectrophoresis with anti-serum protein antibodies. Before use in crossed affinity immunoelectrophoresis or in immunoblotting protein solutions were dialysed overnight at 4 °C against electrophoresis buffer (0.1 M glycine, 0.026 M Tris (pH 8.6)) containing 15 mM NaN_3. IgG and IgA concentrations were determined by laser nephelometry on a Behring nephelometer.

Erythrocyte membranes were prepared by cold hypotonic lysis (5 mM phosphate, 2 mM EDTA (pH 7.4)) as described by Dodge [11] after washing the red blood cells 3–4 times in 154 mM NaCl or 116 mM phosphate (pH 7.4). Filters for leukocyte removal (Imugard IG 500 from Terumo or Ultipor SQ40S from Pall) were employed according to the manufacturers instructions. In some experiments proteolysis was inhibited during preparation by including 2.5 mM PMSF, 5% (v/v) aprotinin, 5 mM *N*-ethylmaleimide, 1.5 μM pepstatin A and 1 mM iodoacetamide in the lysis buffer. These membrane preparations were always more pink than those prepared without proteinase inhibitors. The membrane protein concentration was determined spectrophotometrically in 0.2 M SDS at 278 nm [12].

Dimyristoylphosphatidylcholine (DMPC)-induced vesiculation of red blood cells was performed according to Ref. 13 with minor modifications. Washed erythrocytes were incubated on a shaking table at 30 °C for 4 h at 1 : 10 in a DMPC-solution (0.56 mg/ml in preheated (25 °C) 144 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 7.4), which was sonicated with a Branson B-12 sonifier at 50–60 W until the temperature rose to 45 °C). After centrifugation at $1000 \times g_{av}$ at 4 °C for 20 min the supernatants were centrifuged for 20 min at 4 °C at $36\,000 \times g_{av}$ and then overlayed 5–25% (w/v) continuous Dextran T70 gradients (1 ml to 25 ml gradient solution). After centrifugation at $81\,000 \times g_{av}$ in a SW28 rotor for 18 h at 4 °C (Beckman L8-70 centrifuge) the haemoglobin-rich vesicle layer was harvested by pipetting and washed in the incubation buffer. Membranes and vesicles were stored at −20 °C.

Membranes were solubilized at 2 mg/ml in electrophoresis buffer containing either 1% (v/v) Triton X-100, 2% (v/v) Tween 20, or 1% (v/v) Lubrol PX, sonicated [10] and centrifuged at 30 psi ($122\,000 \times g_{av}$) in a Beckman airfuge for 20 min at 4 °C.

Crossed immunoelectrophoresis and crossed affinity immunoelectrophoresis of solubilized erythrocyte membrane proteins in 1% (w/v) agarose gels containing either 0.1% (v/v) Triton X-100, 0.5% (v/v) Tween 20 or 1% (v/v) Lubrol PX in the electrophoresis buffer were performed according to [10]. In some experiments the rabbit anti-human erythrocyte membrane antibodies were absorbed with human serum albumin to remove anti-albumin activity [14].

Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed in 16 cm slab gels (1 mm thick) according to Fairbanks [15] (5.6 T, 3.6 C). Samples were solubilized and reduced by boiling for 3 min with 40 mM DTT in 50 mM Tris-HCl (pH 8.0) containing 1.0% (w/v) SDS and Pyronin G marker. In some experiments samples were alkylated after solubilization with 60 mM *N*-ethylmaleimide (final concentration). Electrophoresis took place at 22 °C in a Bio-Rad Protean II apparatus at 40 mA/gel. Gels were silver stained according to Heukeshoven [16] followed by fixation in 40% (v/v) ethanol and drying between dialysis films.

Blotting of the SDS-electrophoresis gels by means of semidry electrotransfer took place at 0.8 mA/cm^2 for 1 h as described [17]. Blots were blocked, washed, stained for protein with Amidoblack or probed overnight at room temperature on a shaking table with various dilutions of primary reagents (autologous immunoglobulins) in washing buffer and developed with alkaline phos-

phatase-conjugated secondary antibodies exactly as described [17].

Results

The crossed immunoelectrophoretic reference pattern of immunoprecipitable antigens in a Triton X-100 solubilisate of human erythrocyte membrane proteins is shown in Fig. 1A. The identity of the precipitates was established previously [18,19]. Besides antibodies against membrane proteins the rabbit antibody preparation in the second-dimension gel contains anti-albumin antibodies yielding a precipitate with the albumin added to the membrane solution. This precipitate serves as a marker of the migration in the first dimension of the affinity electrophoretic experiments shown in Figs. 1C, 1D, and 3. Crossed immunoelectrophoresis with different amounts of immunoglobulin incorporated into the first dimensions (constant voltage and time) showed no influence on the albumin migration (not shown). In contrast, certain membrane protein-specific precipitates changed positions when immunosorbent-purified autologous IgG was present during the first dimension electrophoresis (Fig. 1D).

The affected precipitates corresponded to spectrin (d in Fig. 1), band 3 complex (c), and ankyrin (e). The migration of the other membrane proteins visualized in this system, viz. glycophorin (f), haemoglobin (b), and acetylcholinesterase (a) was unchanged. The same pattern of inhibition of migration was seen when protein A [8,9] or protein G-purified (not shown) autologous immunoglobulin fractions were applied.

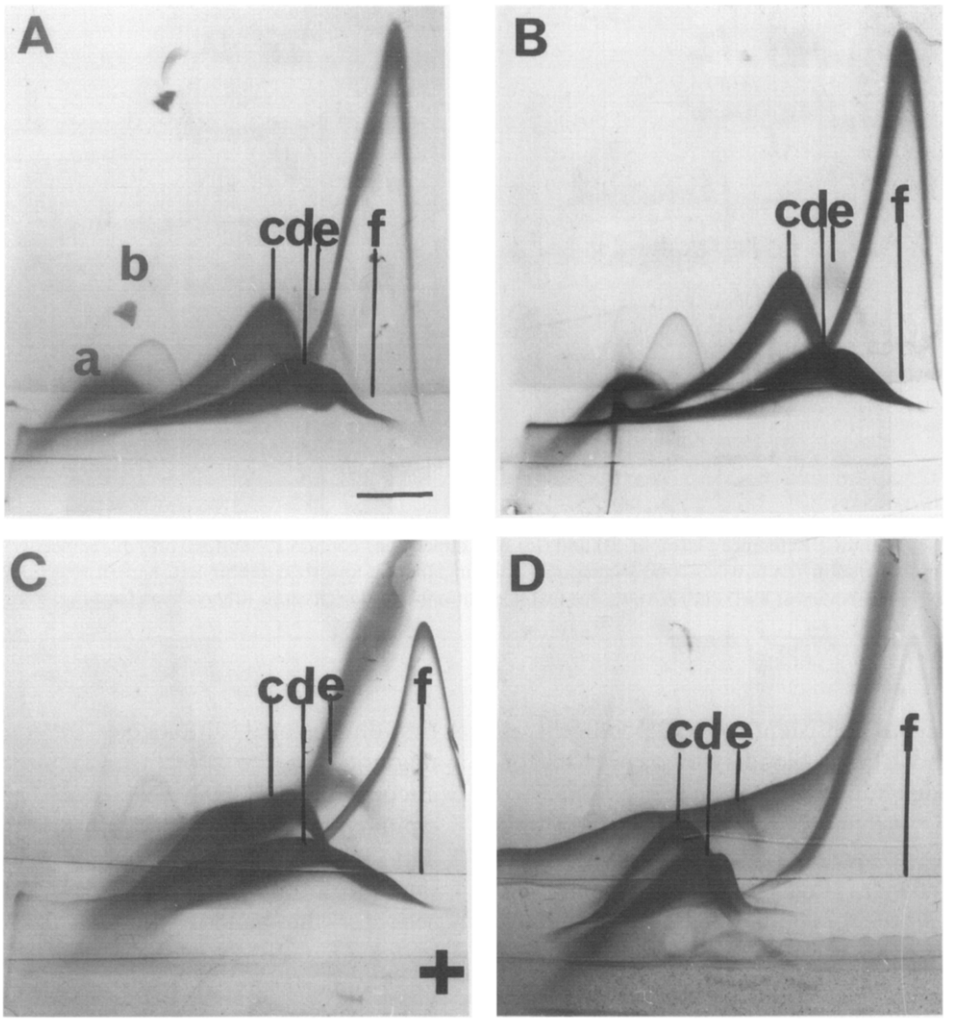

Fig. 1. Crossed immunoelectrophoresis of Triton X-100-solubilized erythrocyte membrane proteins. (A and C) References; (B) contains purified IgA (0.5 mg/ml), and (D) contains immunosorbent-purified autologous IgG (0.8 mg/ml) in the first dimension electrophoresis. Antigen: 10 μl human erythrocyte membrane proteins (2 mg/ml) solubilized with 1% (v/v) Triton X-100. The intermediate gels are empty. The second-dimension gels contain unmodified (C and D) or albumin-absorbed (A and B) rabbit anti-human erythrocyte membrane proteins antibodies at 8 μl/cm^2. Designations: a, acetylcholinesterase (AchE); b, haemoglobin (Hb); c, band 3 protein complex (B3); d, spectrin (sp); e, band 2.1 protein (ankyrin) (2.1); f, glycophorin (Gp). In (C) and (D) f marks the position of the albumin (Alb) migrational marker. a and b are only marked on (A) for the sake of clarity. The location of the anode (+) for the first dimension electrophoresis has been indicated on (C). The bar = 1 cm.

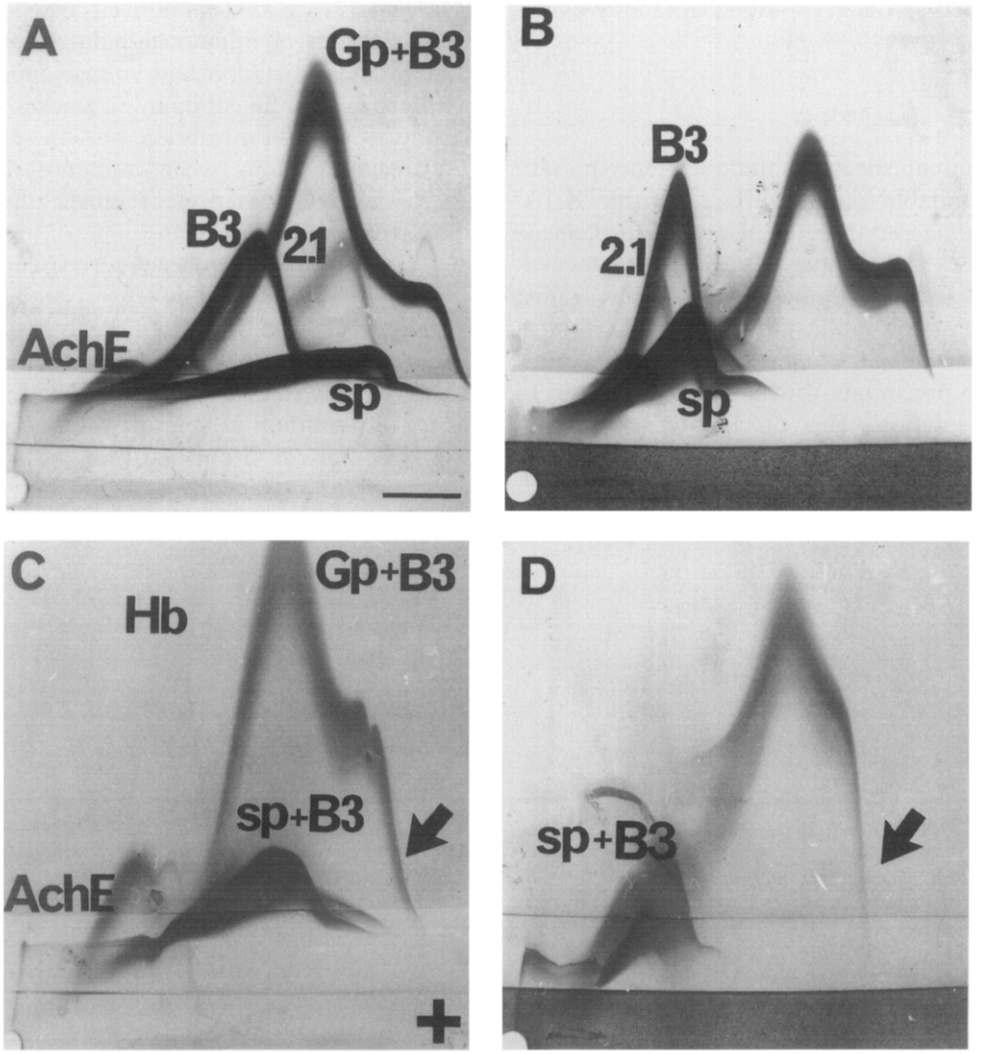

Fig. 2. Crossed affinity-immunoelectrophoresis of Lubrol PX (A, B) and Tween 20 (C, D)-solubilized human erythrocyte membrane proteins (cf. Materials and Methods). (A and C) Reference plates. In (B) and (D) first dimensions contain 1.2 and 1.0 mg/ml, respectively of IgG in autologous protein A-purified immunoglobulin fractions. Second dimensions contain albumin-absorbed rabbit anti-human erythrocyte membrane proteins antibodies at 8 μl/cm^2. Arrows, see text. Conditions and designations otherwise as for Fig. 1.

The observation was consistent for red blood cells of different ABO- and Rhesus blood types as well as for allogeneic immunoglobulin preparations.

Incorporation in the first dimension of purified IgA from the serum of a myeloma patient in comparable amounts gave no changes (Fig. 1B) and the protein-rich effluent from the protein G-column had no effect either (not shown). Removal of leukocytes from the unprocessed blood by means of commercially available filters prior to further handling and the inclusion of a proteinase inhibitor cocktail in the lysis- and washing buffers did not influence the observations.

By solubilization and analysis in presence of alternative non-ionic detergents such as Lubrol PX and Tween 20 (Fig. 2) [20] other precipitation patterns of erythrocyte membrane proteins can be obtained. The effect of first-dimensional autologous immunoglobulin on the migration of the proteins was also seen under these circumstances. Thus, the position of the spectrin precipitate became more cathodic in both systems (Figs. 2B and 2D) when electrophoresis took place in presence of immunoglobulin. The cathodic displacement was also seen for the band 3 precipitate (B3) of the Lubrol system (Fig. 2B) while the changes of the complex band 3-glycophorin precipitates (Gp + B3) were more subtle. A retardation of the front of these precipitates (cf. Fig. 2C and 2D, arrows) indicated an interaction.

The reactivity was further analysed by using dimyristoylphosphatidylcholine-derived vesicles of red blood cells in the crossed affinity immunoelectrophoresis system (Fig. 3). The pure form of band 3 protein obtained by this procedure [13] interacted with autologous im-

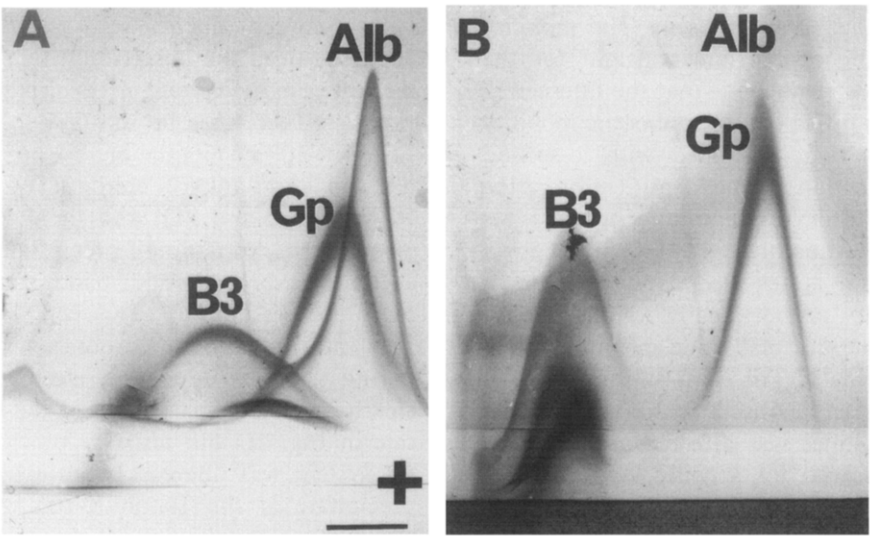

Fig. 3. Analysis of DMPC-derived erythrocyte membrane vesicles. Crossed immunoelectrophoresis of 15 μg Triton X-100-solubilized vesicle proteins with the addition of 1 μl albumin (0.25 mg/ml) were performed without (A) and with (B) 1.2 mg/ml autologous IgG in a protein A eluate incorporated into the first dimension electrophoresis. Content of rabbit anti-membrane protein antibodies in second dimension: 5 $\mu l/cm^2$. Conditions and designations otherwise as for Fig. 1.

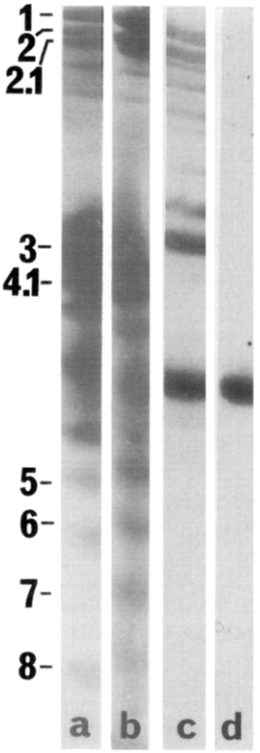

Fig. 4. Reactivity of SDS-gel electrophoresis-separated erythrocyte membrane polypeptides with autologous immunoglobulins in immunoblotting. The electrophoresis of 3.2 μg (a) or 8 μg (b–d) erythrocyte membrane proteins and subsequent electroblotting to nitrocellulose (b–d) were performed as described in Materials and Methods. Lane a, silverstained SDS-gel electrophoresis profile; b, Amidoblack-stained blot; c, blot incubated with a 1:100 dilution of autologous protein A eluate; d, blot without primary reagent. Secondary incubations for lane c and d: rabbit anti-human IgG (1:2000). Designation of bands after Fairbanks et al. [15].

munoglobulins in the first dimension electrophoresis. The ensuing cathodic shift in position of the precipitate appears from the figure.

Interactions were also studied by immunoblotting (Fig. 4). SDS-polyacrylamide gel electrophoresis-separated erythrocyte membrane proteins were probed with dilutions of autologous immunoglobulin fractions. Comparison of an Amidoblack-stained blot (lane b) with the pattern obtained after incubation with the autologous immunoglobulins (lane c) reveal binding to the spectrins (band 1 and 2), ankyrin bands (bands 2.1, 2.2, and 2.3), and to two distinct bands at and above the band 3 region. Also binding to actin (band 5) and reaction in the band 4 to band 5 area was seen on some blots (not shown). The patterns were unaffected by addition of 60 mM *N*-ethylmaleimide to the samples (not shown). Treatment of blots with 5 mM *N*-ethylmaleimide for 15 min before incubation with primary reagents or additional blocking with 5% normal rabbit serum [21] for 30 min did not alter the profile of immunoblot reactivity either (not shown). Membranes prepared in presence of proteinase inhibitors gave equal results. The secondary enzyme-conjugated anti-antibodies sometimes reacted directly with the membrane proteins as it is seen in Fig. 4, lane d, where the first layer reagent is omitted.

Discussion

Affinity electrophoretic techniques rely on measurements of the changes in electrophoretic mobility of a ligand-acceptor complex during electrophoresis where

different amounts of ligand are present. The mobility changes depend on the equilibrium constant for the complex formation. A perequisite is that the interacting molecules in free form differ in electrophoretic mobility [22]. The principle is here combined with a second dimension immunodetection for the identification of the individual human erythrocyte membrane proteins. As the electrophoretic migration of human IgG at pH 8.6 is around zero interaction is revealed as a decrease in electrophoretic mobility proportionally to the amount of IgG. Fig. 1 demonstrates that some membrane proteins extracted by means of Triton X-100 bind autologous IgG because their migration as judged by the position of the precipitates was affected as described. Interactions were observed for spectrin, ankyrin, and band 3 protein which generated precipitates which were displaced towards the cathode.

To investigate if this autoreactivity might be due to proteolysis during the isolation of membranes, removal of leukocytes (which are rich in proteinases) [23,24] by means of filters [25] and inclusion of a cocktail of proteinase inhibitors in the buffers were employed. The precipitation patterns of these membrane preparations were indistinguishable from membranes prepared without precautions against proteolysis except that the preparations with proteinase inhibitors always contained more haemoglobin. The affinity electrophoretic experiments were also independent on this inhibition of proteolysis. Pepstatin selectively inhibits band 3 and glycophorin degradation while *N*-ethylmaleimide also inhibits transglutaminase-induced polymer-formation [23] so none of these phenomena seems to be involved in the generation of autoreactive epitopes. To exclude degradation during electrophoresis the plasmin inhibitor aprotinin [26] was added at 5% to the immunoglobulin fraction. This did not affect the observed reactivity.

The IgG-specificity of the interaction was indicated by experiments where purified IgA was incorporated during the first-dimension electrophoresis with no influence on the electrophoretic mobility of the proteins (Fig. 1). Unspecific protein–protein interactions, e.g., between immunoglobulin molecules and hydrophobic parts of the membrane proteins thus do not seem to contribute to the observations.

Identification of epitopes in crossed immunoelectrophoresis can be complicated by complex formation and coprecipitation [27]. One example is the band 3-containing precipitate in the Triton X-100 system which is a complex containing both band 3 and ankyrin epitopes [19]. Therefore, the binding sites were investigated in more detail using different classes of non-ionic detergents and cytoskeleton-depleted membrane vesicles (Figs. 2 and 3). With Lubrol PX (Figs. 2A and 2B) a more clear delineation of the ankyrin (2.1) precipitate was obtained (compare with Fig. 1A). Sharing of epitopes between ankyrin and band 3 precipitates was apparent from the observation that the cathodic leg of the ankyrin precipitate never crossed the band 3 precipitate. Therefore, in this system as well as in the Triton X-100 system the band 3 precipitate is a complex of band 3 and ankyrin epitopes [19]. In both the Tween 20 (Figs. 2C and 2D) and the Lubrol PX-system the glycophorin-containing precipitate (Gp + B3) was asymmetric because both band 3 and glycophorin epitopes are present [20]. This compound precipitate was also influenced by incorporation of autologous IgG during the first dimension electrophoresis. It is most clearly seen as a sharpening of the front of the precipitate in Fig. 2D but also the precipitate in the Lubrol PX-system was altered. Because the pure glycophorin precipitate of the Triton X-100 system never reacted while the composite band 3-glycophorin precipitate was affected as described above it could be concluded that true band 3 epitopes were involved in the interactions with autologous IgG.

When membrane-derived DMPC-vesicles which are devoid of ankyrin and most spectrin [13] were analysed in presence of Triton X-100 the precipitate of pure band 3 protein also clearly interacted with autologous IgG (Fig. 3B).

To support the findings mentioned above the autoreactivity was also examined by means of immunoblotting from SDS-PAGE (Fig. 4). These experiments demonstrated IgG-associated reactivity against the SDS-treated and dithiothreitol-reduced proteins in protein A-eluates corresponding to the spectrins and to the ankyrin species. In addition, reactivity in the band 4-5 region, and with band 5 (actin) was often observed [9]. No autoantibody reactivity towards band 3 protein could be demonstrated in the immunoblotting experiments shown here.

In some cases the primary level controls (omission of first layer reagent) demonstrated that IgG was present in the sample. The M_r 50000 heavy chain monomer is clearly seen in the control (Fig. 4, lane d). The sharply defined band of about 100,000 which is seen in the band 3 region probably corresponds to the heavy chain dimer of partially reduced immunoglobulin [28]. This band was very weak on the control blot (Fig. 4, lane d) but other blotting experiments with rabbit anti-human IgG as the primary reagent gave strong staining of two bands positioned as is seen here. IgG molecules (alone or in immune complexes) may be firmly associated with the erythrocyte surface and are not washed away during preparation of the membranes. This has also been observed for human platelets [29] and spermatozoa [30]. IgG-opsonization of vesicle material [31] and the amount of IgG bound to intact erythrocytes [32] increase with duration of storage of whole blood. The appearance of membrane-associated IgG in some samples may thus simply reflect the storage time of blood before

processing. Evaluation of this correlation is in progress.

Since binding was seen independent on inhibition of proteolysis in both affinity electrophoresis and immunoblotting, i.e., both under mild as well as under more denaturing conditions it seems that exposure of at least some autoreactive epitopes is not dependent on conformational changes, proteolytic degradation, or cross-linking of membrane constituents.

Affinity electrophoresis can be useful for the estimation of affinity constants [22,33]. By using the figure for the fraction of anti-band 3 reacting autoantibodies given by Lutz et al. [7] and the retardations observed in the experiments above an average functional dissociation constant for the reaction with band 3 is around $1 \cdot 10^{10}$ M. As there may be several autoreactive epitopes on each band 3 molecule reacting with different antibodies of different affinities and since some specific IgG may be lost during immunoglobulin purification this figure is only a rough estimate.

The reactivity with the erythrocyte membrane proteins can be due to cross-reacting epitopes on the individual proteins or represent truly different autoantibody specificities. Several autoantibodies against parts of modified or intact erythrocyte membrane proteins have been described and presumed to be of importance for antibody-mediated sequestration of outdated red blood cells in vivo [6,34,35]. These autoantibodies might be a part of a repertoire of circulating low titer natural autoantibodies against different antigens, including actin [36,37] and spectrin [5,6] which can be demonstrated by means of immunoblotting. Further assessment of autoantibody heterogeneity and the reactions with membrane proteins will be required to evaluate if the interactions described above may be related to the putative immunological recognition of old intact erythrocytes or merely to other tissue-homoeostatic functions [38].

In conclusion, the present study confirms and extends observations based on other experimental approaches [7,24,37] concerning the existence of natural anti-spectrin and anti-band 3 autoantibodies and has, in addition, suggested the presence of autoantibodies against ankyrin. The affinity immunoelectrophoretic analysis in presence of nonionic detergents constitutes a convenient, sensitive, and native approach for the examination of natural autoantibody [38] specificities and is well suited for functional and quantitative studies.

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