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INTERACTION BETWEEN ERYTHROCYTE MEMBRANE PROTEINS AND COMPLEMENT COMPONENTS

II. THE IDENTIFICATION AND PEPTIDE COMPOSITION OF COMPLEMENT COMPONENTS C3 AND C4 DESORBED FROM ERYTHROCYTE MEMBRANES

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SUMMARY

(1) Following lysis of sheep erythrocytes with antibody plus human complement, several serum proteins can be desorbed from washed membranes by incubating these in isotonic buffer at 37 °C. We have analyzed these desorbed proteins by isoelectric focusing combined with sodium dodecylsulfate–polyacrylamide gel electrophoresis and crossed immunoelectrophoresis.

(2) Crossed immunoelectrophoresis using antisera to human serum yields complex patterns of precipitation arcs, but the use of monospecific anti-C3 and anti-C4 allows us to identify these components.

(3) Isoelectric focusing in polyacrylamide followed by sodium dodecylsulfate–polyacrylamide gel electrophoresis at right angles resolves the desorbed serum proteins into three major components with characteristic isoelectric points and electrophoretic mobilities in sodium dodecylsulfate–polyacrylamide gel electrophoresis. One component is serum albumin, non-specifically absorbed. Preparative isoelectric focusing followed by crossed immunoelectrophoresis shows that the other two components comprise desorbed C3 and C4 components of complement. Their isoelectric points are 5.1 and 5.7–6.2 respectively. The desorbed C3 components comprise two entities with apparent molecular weights of 160-180 000. Disulfide cleavage with dithiothreitol splits these into two major components of apparent molecular weight 80 000 and 45 000 and one minor component of 30 000. Desorbed C4 also resolves into two closely associated bands of apparent molecular weight 160 000-170 000 in sodium dodecylsulfate gel electrophoresis. Dithiothreitol treatment reduces these to subunits of apparent molecular weight 80 000, 32 000 and 30 000. Moreover, the appearance of one protein with decreased relative mobility is observed.

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(4) These data are discussed in the light of recent findings based on two-dimensional separations of membrane proteins following complement-mediated lysis. They lead us to conclude that after solubilisation of complement-treated membranes in sodium dodecylsulfate, molecular complexes consisting of membrane-bound C3 and membrane proteins persist in solution. These complexes are stabilized by the intramolecular disulfide bonds of C3.

INTRODUCTION

In a recent communication [1], we presented data on two-dimensional sodium dodecylsulfate–polyacrylamide gel electrophoresis of erythrocyte membranes isolated after complement-mediated cytolysis. We showed that certain earlier-reacting complement components (before C6) can be detected by two-dimensional sodium dodecylsulfate–polyacrylamide gel electrophoresis, and provided evidence that one or more of these components might penetrate the target membrane and bind non-covalently to membrane proteins; this process appears to be stabilized by disulfide bonds within the complement components.

In this paper we identify the major complement components seen by two-dimensional sodium dodecylsulfate–polyacrylamide gel electrophoresis [1] by desorbing the components from membranes and analyzing them by electrophoretic and immunoelectrophoretic methods. We also describe a simple one-step procedure for the preparation of C3 and C4 components desorbed from membranes and present some biochemical data on their molecular structure.

MATERIALS AND METHODS

Unless otherwise stated, we obtain all chemicals and biochemicals from Serva (Heidelberg), Boehringer (Mannheim) and Merck (Darmstadt). Our source of human complement is serum from freshly drawn human blood. Targets for complement action are sheep erythrocytes stored at 4 °C in acid–citrate dextrose. Rabbit anti-sheep red blood cell antiserum and rabbit anti-human C4 monospecific antiserum were generous gifts from Behring (Marburg). Purified rabbit immunoglobulins raised against human serum proteins (SA 100), monospecific anti-human serum albumin and anti-human C3 (β1a and β1c) rabbit immunoglobulins were obtained from Dakopatts (Copenhagen), agarose from Litex (Glostrup, Denmark, Batch AGS 131 A) and ampholines of pH range 3.5–10.0 from LKB (Uppsala, Sweden).

Complement lysis

We lyse sheep red blood cells with antibody and complement as described previously [1]. As controls, we prepare membranes by hypotonic lysis in 5 mM phosphate buffer, pH 8.0 as in ref. 2, incubate these membranes with heat-inactivated complement (serum incubated at 56 °C for 2 h and subsequently centrifuged at 200 000 $g \times$ min). Following treatment of cells/membranes with active/inactive complement in veronal-buffered saline containing 0.00015 M Ca^{2+} and 0.001 M Mg^{2+}, we wash membranes four times in 40 vols of ice-cold 5 mM phosphate buffer and utilize the pelleted membranes in desorption experiments. Membrane protein concentration is determined by the Ninhydrin procedure [3].

Desorption of membrane-bound proteins

We make the concentrated membrane suspensions (protein concentration 4–5 mg/ml) isotonic (300 mosmolar) by addition of five-fold concentrated veronal-buffered saline (1500 mosmolar), incubate for 4h at 37 °C, centrifuge at 9×10^6 $g \times$ min in a Spinco ultracentrifuge (model L2-653, rotor type SW 50.1) and subject the supernatant fluids to electrophoretic and immunoelectrophoretic analyses.

The concentration of protein desorbed from complement-treated membranes and present in the supernate under the given conditions is approximately 1.0–1.3 mg/ml.

Isoelectric focusing

We dialyse samples against 100 vols of 0.5 mM EDTA, pH 7.0 for 2h and then make these 2 % in ampholines and 10 % in sucrose. For isoelectric focusing, we apply 300–400 µl samples containing approximately 0.4–0.6 mg protein to 4 % polyacrylamide gel discs (diameter 0.6 cm. height 13 cm) which are crossed-linked to 2.5 % with *N,N'*-methylenebisacrylamide and contain 1 % ampholines and 10 % sucrose. The gels are prepared as in [4] except that distilled water is substituted for urea. Isoelectric focusing takes place in a cold room at 4 °C. We pre-electrophorese at 0.75 mA per gel for one hour to remove residual ammonium persulfate. After application of samples, we focus at a constant current (0.75 mA/gel) until the voltage is 40 V/gel. Electrofocusing is then continued at this voltage for another 12h. We measure pH gradients by sectioning gels into 5 mm portions, soaking these in degassed, distilled water for one hour at 4 °C, and measuring the pH of the samples.

Isoelectric focusing linked to sodium dodecylsulfate–polyacrylamide gel electrophoresis

We employ a multislab polyacrylamide gel electrophoresis apparatus for two-dimensional studies [5]. After isoelectric focusing, we extract the gels from the glass tubes, dialyze these against electrophoresis buffer containing 3 % sodium dodecyl-sulfate for 60 min at room temperature and then attach them horizontally on to polyacrylamide gel slabs for second-dimensional runs [5]. However, we have slightly modified the procedure for polymerizing gel discs on to the gel slabs, and now use a solution of 1 % agarose in electrophoresis buffer heated to 60 °C to attach first-dimensional gels on the polyacrylamide gel slabs.

In order to analyze the effects of disulfide cleavage, we also dialyze extracted gels against buffer containing 3 % sodium dodecylsulfate and 40 mM dithiothreitol before running in the second dimension.

Immunoelectrophoresis

We perform crossed immunoelectrophoresis combined with intermediate gel techniques [6, 7] using a Multiphor LKB apparatus model 2117. The electrophoresis buffer is barbital buffer, pH 8.7, of ionic strength 0.02 [6]. First-dimensional runs are at 10 V cm^{-1}. We use albumin-absorbed bromphenolblue as a marker and allow this to migrate 6.5 cm. Second-dimensional runs are on 8 · 10 cm agarose plates at 2 V cm^{-1} for 16 h. The thickness of the agarose layer is 1.5 mm and 1.0 mm for first and second-dimensional runs, respectively.

Immunoelectrophoresis after electro-focusing

Following isoelectric focusing in polyacrylamide, one can directly visualize the main protein bands as white precipitates. We cut out selected portions of the gels, homogenize them by forcing through 2 ml syringes, and add approximately $\frac{1}{2}$ volume of veronal-buffered saline to each sample. After allowing the buffer to diffuse for 3 hours at 4 °C, we directly apply approximately 150–200 μl portions of homogenized gel to agarose plates and proceed with crossed immunoelectrophoresis. We also subject aliquots of these samples to sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis

We make gel portions obtained after isoelectric focusing 3 % in sodium dodecylsulfate–polyacrylamide gel electrophoresis and 10 % in sucrose, allow sodium dodecylsulfate to diffuse for one hour at 4 °C, subsequently heat for 2 min at 100 °C and perform sodium dodecylsulfate–polyacrylamide gel electrophoresis on polyacrylamide gel discs (0.5 cm in diameter and 10 cm in height) as in [8] using 5 % gels crossed-linked to 2.5 % with *N,N'*-methylenebisacrylamide. In addition, aliquots of all samples are made 40 mM in dithiothreitol, and incubated at 37 °C for 60 min to produce disulfide cleavage. The electrophoresis buffer is 0.04 M Tris, 0.02 M sodium acetate, 0.002 M EDTA, pH 7.4 containing 1 % sodium dodecylsulfate, and electrophoresis is at 5 mA per gel at room temperature. The tracking dye, Pyronin G, is allowed to migrate 7.5 cm.

Calibration of gels

We calibrate gels as in refs 9 and 10, using rabbit immunoglobulin G (155 000), phosphorylase A (93 000), bovine serum albumin (69 000), egg white albumin (45 000), bovine pancreas α-chymotrypsinogen A (25 700), horse skeletal muscle myoglobin (17 200) and horse heart cytochrome *c* (13 500) as standard proteins.

Staining procedures

We stain polyacrylamide gels with Coomassie Brilliant Blue as in [8] and agarose plates as in [6]. Following isoelectric focusing and subsequent sodium dodecylsulfate–polyacrylamide gel electrophoresis, it is necessary to first elute the diffusely migrating ampholines from the gels. For this, we have modified our original procedure [4] and now fix and wash gels in 50 % methanol rather than in 25 % isopropanol/10 % acetic acid. Methanol enables one to elute the ampholines faster than isopropanol/acetic acid: the latter causes reversible precipitation of ampholines in the gels. With 50 % methanol, 12 h of washing suffice to elute ampholines before staining with Coomassie Brilliant Blue.

Experimental protocol

We perform isoelectric focusing of identical samples on five gels and subject two gels to two-dimensional polyacrylamide gel electrophoresis plus and minus cleavage of disulfide bonds in the second dimension. We slice one gel to determine the pH gradient. The fourth gel is stained, and the last gel is used to link isoelectric focusing with immunoelectrophoresis. Aliquots of the fractions from the last gel are subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis.

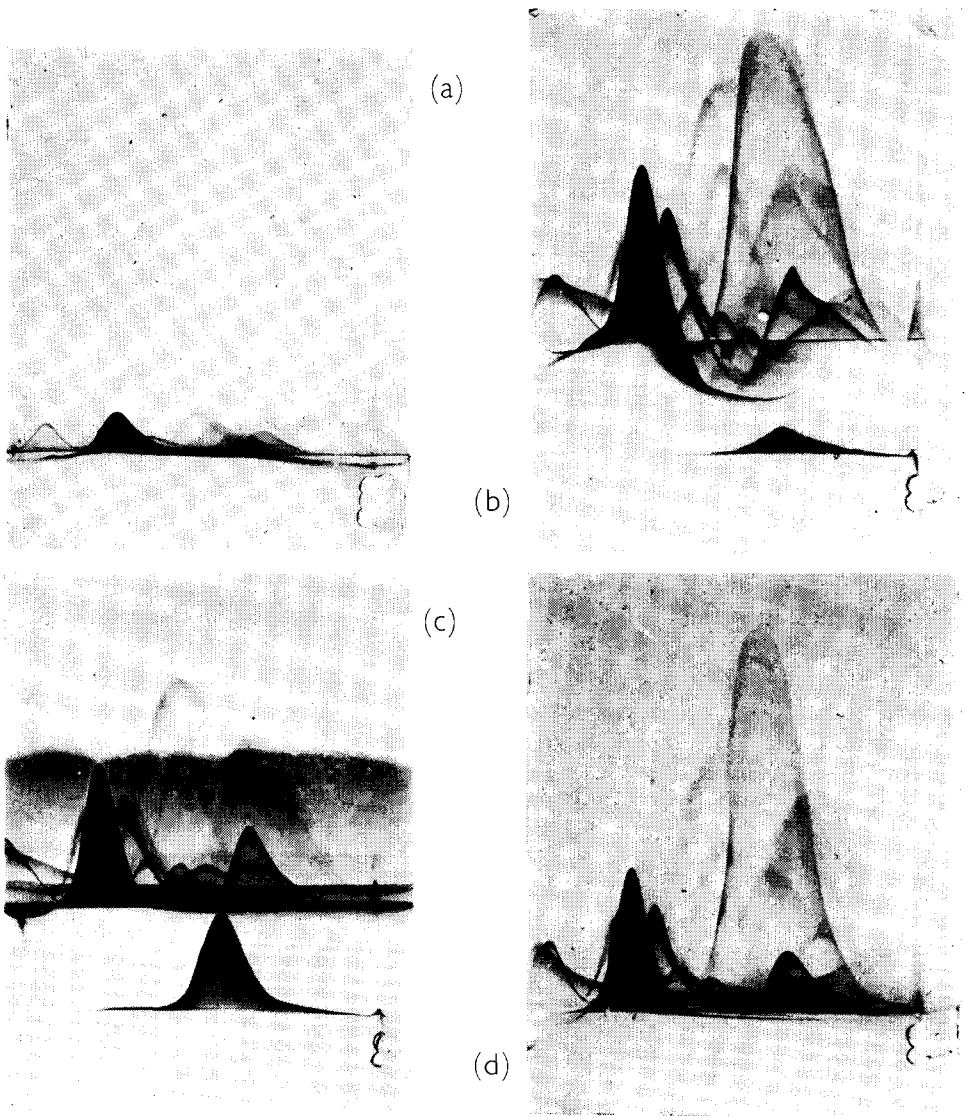
Photography

We photograph gel discs and slabs and agarose plates with a Polaroid Land MP-3 camera using a Polaroid film type PN 55. Shutter settings are 11/16 or 16/22. Exposure time is one second.

RESULTS

Immunoelectrophoresis of desorbed proteins

When hypotonically lysed control membranes are incubated with inactivated complement, a number of serum proteins absorb on to the membranes and cannot



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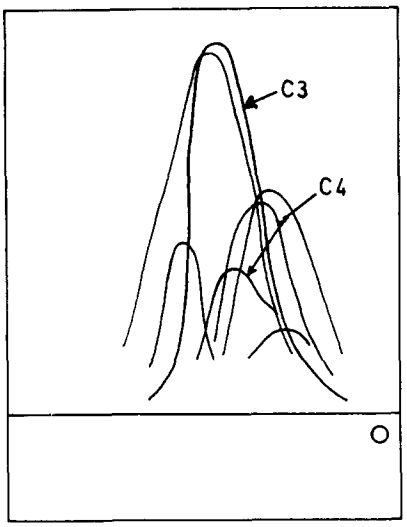

Fig. 1. Crossed immunoelectrophoresis of the control and complement eluates. Applied volume: 40 μl. 600 μl antibodies to whole human serum incorporated in the gels. (a) Control eluate. (b) Complement eluate. (c) Complement eluate, 100 μl, anti-C3 incorporated into the intermediate gel. (d) Complement eluate, 100 μl anti-C4 incorporated into the intermediate gel. (e) Schematic illustration of the main complement-specific precipitations; arrows point to C3 and C4 components.

be removed by washing with 5 mM phosphate. They can be partially desorbed by incubation of membranes in isotonic buffer at 37 °C. Analysis of the desorbed proteins by crossed-immunoelectrophoresis yields characteristic precipitation patterns with antisera to whole human serum (Fig. 1a). In contrast, no precipitations are found when rabbit antiserum to sheep erythrocyte membranes is incorporated into the gels. After the action of complement on membranes, we find that the number of desorbed proteins increases, resulting in the appearance of new precipitation arcs (Fig. 1b). We shall henceforth refer to these eluates as the control eluates, and the complement eluates, respectively.

Incorporation of either monospecific anti-C3 or anti-C4 into an intermediate gel demonstrates the presence of these components in the complement eluates only (Fig. 1c and 1d). Corresponding precipitation arcs disappear from the reference pattern in the upper gel. Fig. 1e schematically shows the main, complement-specific precipitates; the identified C3 and C4 components are pointed out with arrows.

Preliminary kinetic studies indicate that desorption of proteins nears a maximum after 4–5 h at 37 °C. However, detailed immunoelectrophoretic studies now underway are necessary to establish whether there are quantitative differences in the desorption rates of individual protein components.

Desorption of serum proteins at lower temperatures also takes place, but at progressively slower rates. Also, it is important to desorb in isotonic buffer, because decrease in ionic strength of the desorbing buffer leads to membrane protein extraction, which hampers subsequent analyses described below.

Isoelectric focusing

Fig. 2 depicts the isoelectric focusing pattern of the complement eluate together

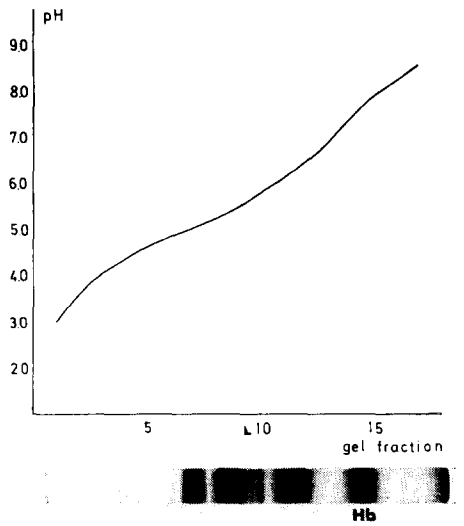

Fig. 2. Isoelectric focusing of the complement eluate and the corresponding pH gradient. Hb: hemoglobin. Coomassie Brilliant Blue.

with the corresponding pH-gradient. The gradient is satisfactorily linear between pH 3.0–8.5. A number of protein bands is detectable, one set between pH 4.7–5.2, and another discrete set at pH 5.7–6.2. Hemoglobin components focus near pH 7.0 in this system.

Isoelectric focusing linked to sodium–dodecylsulfate gel electrophoresis

Following electrofocusing with sodium dodecylsulfate gel electrophoresis at right angles yields characteristic electrophoretic patterns, as shown in Fig. 3. We have given the corresponding pH-gradients and molecular weight scale as determined by conventional gel calibration methods.

Figs 3a and 3b show the electrophenograms of the control eluates with or without disulfide cleavage prior to running in the second dimension. Two major protein components are visualized, but some faint peptide spots which cannot be precisely characterized are also visible on the original gel plates. Also, one protein appears to be unable to penetrate into the gel, and therefore remains at the gel origin during focusing. The two major non-specifically absorbed serum proteins focus at pH 4.8. Their apparent molecular weights are approximately 60 000 and 40 000. After dithiothreitol treatment, there is a change in the relative mobility of both proteins, one migrating in the region of apparent molecular weight 65 000, the other showing an apparent molecular weight of approximately 20 000.

Figs 3c and 3d show the electropherograms of the complement eluate. Without disulfide cleavage before second-dimension (Fig. 4c), two new major components appear, focusing near pH 5.1 and 5.7–6.2. We arbitrarily name the proteins A and B, respectively. Many more additional faint spots are visible; they are spread over a wide pH-range and their apparent molecular weights range between 50 000–130 000. Sodium dodecylsulfate gel electrophoresis resolves protein A into two components of apparent molecular weight 160 000–180 000. Dithiothreitol splits both of these to

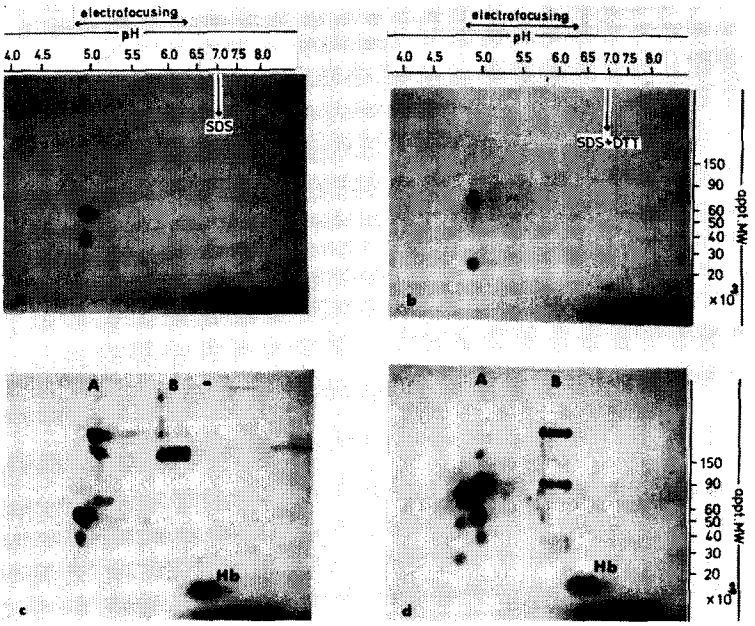

Fig. 3. Two-dimensional separations of the complement and control eluate. Isoelectric focusing: left to right; sodium dodecylsulfate (SDS) gel electrophoresis with and without –S–S cleavage: top to bottom. (a) Control eluate. (b) Control eluate with –S–S– cleavage before second dimension. (c) Complement eluate. (d) Complement eluate with –S–S– cleavage before second dimension. A and B: the two major, complement-specific proteins (details in test). Appt. MW: apparent molecular weight. Hb: hemoglobin. DTT: dithiothreitol. The lake-like material at the bottom of the gels represents uneluted ampholines. Coomassie Brilliant Blue.

yield 3 proteins of apparent molecular weight around 80 000, 45 000 and 30 000 (compare Figs 3c and 3d). The series of bands which we name protein B appears to represent polymorphisms of one molecular species, because all components migrate identically in sodium dodecylsulfate gel electrophoresis. Without disulfide cleavage, this component resolves into a paired set of bands of apparent molecular weight 160 000–170 000, but a complex behaviour is observed after disulfide cleavage (Fig. 3d). Thus, dithiothreitol treatment results in the appearance of a major component with a decreased electrophoretic mobility in sodium dodecylsulfate as compared to the untreated material. In addition, protein B generates three other components of apparent molecular weight 80 000, 32 000 and 30 000.

Isoelectric focusing linked to immunoelectrophoresis

Identification of proteins A and B, as well as the major non-specifically desorbed protein is possible by immunoelectrophoresis of the electrofocused components. For this, we section gels after isoelectric focusing, and follow directly with immunoelectrophoresis and sodium dodecylsulfate gel electrophoresis. The sodium dodecylsulfate gel electrophoresis patterns of the different gel sections correspond to analogous parts of the two-dimensional separation, which one can also use as a reference pattern. Crossed immunoelectrophoresis with monospecific anti-albumin, anti-C3 and anti-C4 in intermediate gels, and antiserum to whole human serum in the uppermost gel now allows identification of the components.

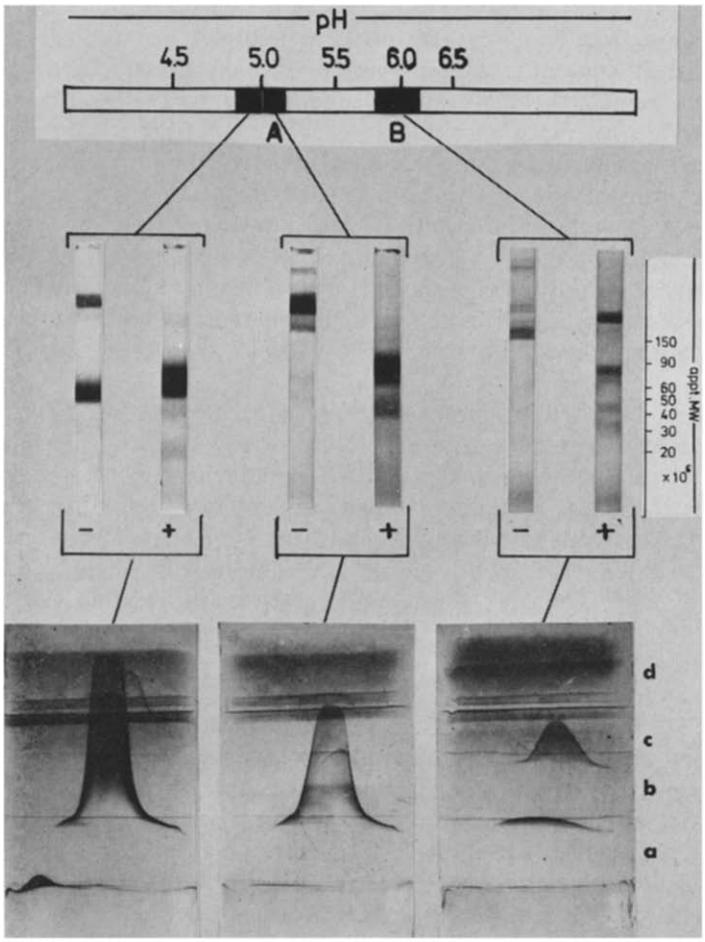

Fig. 4. Isoelectric focusing linked to sodium dodecylsulfate and crossed-immunoelectrophoresis. Above: schematized reproduction of the gel fractions analysed. Middle section: sodium dodecylsulfate gel electrophoresis patterns of the different gel fractions, with (+) and without (−) dithiothreitol. Below: crossed-immunoelectrophoresis of the corresponding fractions with intermediate gels containing the following monospecific antibodies: (a) 100 μl anti-albumin (b) 100 μl anti-C3 (c) 100 μl anti-C4. Uppermost gel (d) contains 150 μl of antibodies to whole human serum. Appt. MW: apparent molecular weight.

As shown in Fig. 4, the pH 4.7–4.9 fraction contains two major proteins of apparent molecular weight 60 000 and 180 000 when analyzed by sodium dodecylsulfate gel electrophoresis. The protein of lower molecular weight corresponds to the major unspecifically absorbed protein also seen in the control eluate. In analogy to the two-dimensional plates (Figs. 3c and 3d), dithiothreitol splits the high molecular weight component into its subunits, and the lower molecular weight protein shifts to a region of slightly lower relative mobility in sodium dodecylsulfate. In crossed immunoelectrophoresis, strong precipitation arcs are obtained with anti-albumin and anti-C3, and a number of minor precipitates are observable in the uppermost gel. There is no precipitation with anti-C4.

Electrophoresis in sodium dodecylsulfate of the pH 5.0–5.2 material reveals only one set of bands at the molecular weight region of 160 000–180 000 (compare also with Fig. 3c): dithiothreitol splits these into the subunits observed in two-dimensional separations (Fig. 3d). Immunoelectrophoresis shows only a trace of albumin, but anti-C3 precipitates strongly, and a faint precipitation is sometimes found in the gel containing anti-C4 when gels are overloaded during electrofocusing. The uppermost gel contains only one minor precipitate. These data allow one to conclude that the protein referred to as protein A in our two-dimensional pattern is desorbed C3, and that the main non-specifically absorbed protein is serum albumin. Our finding that serum albumin shows decreased electrophoretic mobility in sodium dodecylsulfate after dithiothreitol treatment is also in accordance with other electrophoretic data on this protein [11].

Sodium dodecylsulfate gel electrophoresis of material focusing at pH 5.7–6.2 reveals the set of bands corresponding to protein B in the two-dimensional plate (Fig. 3c). Dithiothreitol causes the appearance of the same subunits observed in Fig. 3d. In immunoelectrophoresis, we find a minor precipitation with anti-C3, and one main precipitate in the gel containing anti-C4. There are no precipitations visible in the uppermost gel, indicating the absence of further serum protein contaminants detectable with this antiserum. Thus, protein B can be identified as desorbed C4.

DISCUSSION

Two-dimensional sodium dodecylsulfate–polyacrylamide gel electrophoresis of sheep erythrocyte membrane proteins yields a typical peptide pattern following complement lysis when disulfide cleavage is introduced between first and second dimension. This separation procedure reveals that one or more complement components reacting before C6 are complexed to membrane proteins [1]. The primary objective of the present study was to identify the major complement components visualized by two-dimensional sodium dodecylsulfate–polyacrylamide gel electrophoresis and, in particular, to find out which components of the complement system become attached to membrane proteins during complement lysis.

Because sodium dodecylsulfate grossly denatures proteins and hinders subsequent immunoelectrophoretic studies, we found it more convenient to partially elute the complement components from membranes, perform partial identification by immunoelectrophoresis, and then analyze their electrophoretic behaviour in sodium dodecylsulfate. This makes an indirect identification of the components observed in two-dimensional sodium dodecylsulfate–polyacrylamide gel electrophoresis possible, because proteins of identical electrophoretic mobility are present in complement eluates and on complement-treated membranes.

Fig. 5 illustrates this point more precisely. We compare the two-dimensional sodium dodecylsulfate–polyacrylamide gel electrophoresis pattern of complement-treated sheep erythrocyte membranes (Fig. 5a, see also ref. 1) with the plate in Fig. 3d which is also reproduced schematically (Fig. 5b). In the first case, complement-treated erythrocyte membranes are solubilized in sodium dodecylsulfate and electrophoresed in a first dimension. Following this, the gels are extracted from the tubes, dialysed against dithiothreitol-containing buffer and run horizontally in a second dimension. Typically, high molecular weight complexes (termed A–G) split into

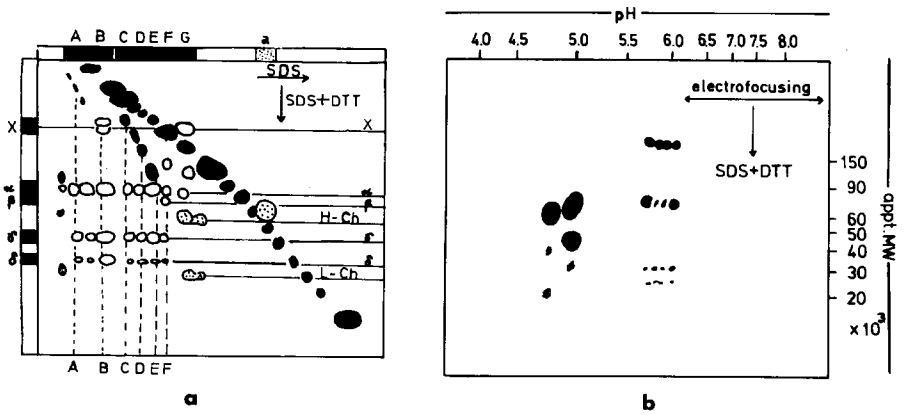

Fig. 5. (a) Two-dimensional electrophoretic pattern of erythrocyte membranes treated with complement (see ref. 1, fig. 4b). (b) Schematic reproduction of the main protein spots of plate 3d. Open circles in 5a: non-membrane proteins, part of which are attached to membrane proteins (closed circles). Dotted circles: non-specifically attached serum proteins (serum albumin and immunoglobulins, see ref. 1). SDS: sodium dodecylsulfate; DTT: dithiothreitol; appt. MW: apparent molecular weight.

individual components as illustrated in Fig. 5a [1]. In our first work, we were able to identify membrane proteins by labelling these with dansylchloride [1]. The results indicated that the proteins illustrated as empty circles in Fig. 5a were probably complement proteins, because these spots did not fluoresce when membranes were labelled with dansylchloride prior to complement treatment [1].

In the present study, desorbed complement components could be separated by isoelectric focusing and characterized molecularly by sodium dodecylsulfate gel electrophoresis in a second dimension. Fig. 5b illustrates the separation pattern when second dimensional runs are performed with disulfide cleavage. The separation system in the second dimension is identical to the one in Fig. 5a, and identical proteins will therefore show identical migration patterns. We now find that the protein termed "X" in the previous work corresponds to the high molecular weight C4 component, whereas peptides α, γ, δ correspond exactly to the subunits of C3 found in the present study. We therefore conclude retrospectively that activated C3 forms complexes with membrane proteins. Our first work demonstrated the stability of such complexes in sodium dodecylsulfate. Despite this, we find that membrane-bound C3 is gradually released into the fluid phase in isotonic buffer at 37 °C. Two-dimensional sodium dodecylsulfate gel electrophoresis of complement-treated membranes (performed as in ref. 1) after desorption of complement proteins has shown that desorption of the C3 component is not visibly selective, i.e. the protein spots representing the C3 subunits and the corresponding membrane protein spots all become fainter in intensity. This indicates that dissociation of C3 from membraneproteins takes place in our desorption experiments. We suspect that this release of membrane-bound C3 might involve some active process, which is interrupted by sodium dodecylsulfate. At this stage, we emphasize that one should not refer to the membrane proteins observed to be bound with C3 as "C3-receptors", as this term might be taken to imply certain biological specificities which are as yet unestablished.

Methods for the preparation of native C3 have been detailed [12, 13] and the

main biochemical changes undergone by this molecule during its activation and attachment to target membranes are also known [12, 14, 15]. Thus, it is now well established that only activated C3, also termed C3b, binds to membranes during the complement reaction [12, 15–17]; accordingly, the C3 component which we desorb from complement-treated membranes must be derived from C3b. However, C3b and the desorbed C3 component which we obtain need not be molecularly identical, because secondary degradation of membrane-bound C3b by proteolytic serum enzymes such as the C3b inactivator [18] cannot be excluded in our experimental system. Therefore, the "subunits" of membrane-desorbed C3b which we find may not be identical to the original subunits of native C3b. Nevertheless, the data do show that C3b possesses at least one intramolecular disulfide bond. Our first work indicated that these disulfide bonds were important in stabilizing membrane protein/complement protein complexes. In this context, it is of interest to note that dithiothreitol also totally destroys C3 binding capacity to target membranes [19]. Methods for the preparation of C4 are also well established [20], and the biochemical reactions of this complement component have been studied in detail [21, 22]. Cleavage of the native molecule into its active fragment, C4b, has been demonstrated [23, 24]. Again, the C4 component which we desorb must therefore be derived from C4b. Analytical ultracentrifugation studies indicate that C4b has a molecular weight of about 200 000 [23]. Our electrophoretic system reveals a strange electrophoretic behaviour of our desorbed C4 component in sodium dodecylsulfate. Without disulfide cleavage, this molecule has an apparent molecular weight of approximately 160 000, a value considerably lower than reported for native C4b by others [23]. This could be an indication that the C4 component which we obtain represents a degradation product of C4b. We also find some high molecular weight aggregates of desorbed C4 in sodium dodecylsulfate gel electrophoresis which could be explained by earlier findings that activated C4 has a tendency to form molecular aggregates [21]. Dithiothreitol liberates "subunits" of apparent molecular weight 80 000, 32 000 and 30 000, but we constantly observe the appearance of a molecule possessing a decreased relative mobility as well. Further studies are necessary to explain these findings. Again, we stress that, as in the case of C3b, we may not be observing the original C4b subunits, because our experiments do not exclude secondary degradation of membrane-boundC4b.

The C4 component which we desorb focuses as a series of bands around pH 5.7–6.2. Differences in the two-dimensional pattern of this molecule are observable when complement from different donors is used. This finding is consistent with other data on C4 polymorphism, which can also be detected by crossed-immunoelectrophoresis [25, 26].

The precipitation pattern of the complement supernate in crossed-immunoelectrophoresis indicates that there are other complement proteins in the eluates which remain to be identified. However, we cannot exclude the possibility that certain serum proteins lose their ability to bind non-specifically to membranes after heating at 56 °C. These would therefore not be detected in the control eluates.

It is of interest to observe that membrane-bound C3b and C4b or derivatives of these can be partially eluted from target membranes in relatively large quantities, provided that a surplus of complement is used for lysis. This offers an opportunity to prepare these components in a one-step procedure, because electrofocusing effects excellent separation. On the other hand, desorbed C3b and C4b components cannot

be separated by simple sodium dodecylsulfate–polyacrylamide gel electrophoresis because of their similar molecular weights. Whereas our isolation procedure does not yield hemolytically active complement components, it may be useful for the isolation of C3b and C4b derivatives for immunization experiments because it circumvents the difficult preparative steps necessary for the isolation of C3 and C4 from whole serum. The general principle of using membranes as "affinity absorbents" as in this study could prove to be a useful tool in other fields of immunobiological research in the future.

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