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IMMUNOCHEMICAL ANALYSES OF MEMBRANE-BOUND COMPLEMENT

DETECTION OF THE TERMINAL COMPLEMENT COMPLEX AND ITS SIMILARITY TO "INTRINSIC" ERYTHROCYTE MEMBRANE PROTEINS

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SUMMARY

(1) Membranes of sheep erythrocytes lysed with antibody and human or rabbit complement were solubilized in non-ionic detergents (Triton X-100 or Berol EMU-043) and analysed immunochemically using antisera directed against individual complement components. The precipitation behaviour of membrane-bound C3, C5, C6 and C9 components of complement was examined by immuno-double diffusion, rocket- and crossed immunoelectrophoresis performed in agarose gels containing 1 % non-ionic detergent.

(2) Membrane-bound C5, C6 and C9 are antigenically altered compared with the native (serum) components.

(3) Immuno-double diffusion in the presence of non-ionic detergents reveals formation of C5-C6-C9 complexes on the membranes; these complexes are stable in non-ionic detergent. No complex formation was detected in serum between native C5, C6 and C9 components. There was also no evidence for complexing between membrane-bound C3, C4 or membrane proteins and the "late-reacting" complement components.

(4) The extractability of complement components by various manipulations has been studied by use of quantitative rocket immunoelectrophoresis. Up to 65 % of membrane-bound C3 is readily extracted by dialysis of membranes against 1 mM EDTA, pH 8.0, 100 mM EDTA, pH 8.0, 1.2 NaCl plus or minus EDTA, by extraction in isotonic buffers at 37 °C, by heating at 45 °C over several hours, or by treating membranes with 1 mM *p*-chloromercuribenzoate sulfonate. In contrast, less than 6 % of the terminal complement complex can be eluted by any of the described methods or combination of methods.

(5) Our data suggest that the terminal complement complex associates with membrane "core" components through apolar interactions.

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INTRODUCTION

Over the past 15 years, increasing data has accumulated on the molecular nature of individual complement components and their reactions during the course of assembly on target cell surfaces [1,2]. It is now well established that of the known nine protein components comprising the complement system, only the last five, termed C5–C9, are required for the disruption of cell membranes [1–6]. Recent publications have dealt with the modes of interaction in free solution among these five and have also presented evidence that complexes, similar to those that form in solution, exist on erythrocyte membranes isolated after complement-mediated cell lysis [1, 5–7]. However, the interactions between the terminal complement components associated with the membrane and diverse membrane entities remain unknown. Moreover, data on possible changes in the molecular composition and/or organization of target membranes accompanying complement-induced lysis are scarce indeed. Most studies in this area have focused on possible modifications in phospholipid structure/composition in target membranes, but to date no unambiguous evidence for such lipid changes has been obtained [8–10].

We have therefore carried out experiments designed to analyse the complement components bound to target membranes and to uncover possible changes in membrane structure and/or composition during complement lysis. In our initial reports [11, 12], we documented the utility of two-dimensional detergent-gel electrophoresis methods in analysing the associations of early-reacting complement proteins with erythrocyte membranes. Also, we showed by freeze-fracture electron microscopy that the terminal actions of complement alter the "core" structure of sheep erythrocyte membranes [13]. Finally, we have recently [14] documented the utility of immunoelectrophoresis in the presence of non-ionic detergents [15–17] for the analysis of membrane-bound complement components.

This communication extends our immunochemical analyses of membrane-bound complement components. We describe the precipitation behaviour of membrane-bound components C3, C5, C6 and C9 in immuno-double diffusion, rocket and crossed immunoelectrophoresis and, by these techniques, demonstrate that terminal complement proteins form complexes on target membranes. Finally, we demonstrate the differential extractability of complement components under various conditions by use of quantitative rocket immunoelectrophoresis.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals and biochemicals were obtained from Serva (Heidelberg), Boehringer (Mannheim) and Merck (Darmstadt). Sheep erythrocytes stored in acid/citrate/dextrose for 4–21 days were used in all experiments. Fresh human or rabbit serum were our sources of complement. Rabbit anti-sheep erythrocyte antiserum (amboceptor) and monospecific antisera against human complement components C4 (batch 2247 AD) and C9 (batch 2602 M) and monospecific goat antiserum against human C5 (batch 2220 M) were purchased from Behring (Marburg). Monospecific rabbit anti-human C3 (β1a and β1c) immunoglobulins were a generous gift of Dakopatts A/S, Copenhagen. Monospecific anti-rabbit C6 anti-sera, which cross-react with human C6, were raised in C6-deficient rabbit strains (18, 19) by in-

jecting normal rabbit serum in complete Freund's Adjuvant into C6-deficient rabbits.

Agarose was purchased from Litex (Glostrup, Denmark) (batch Ags 155) and l'Industrie Biologique française (Indubiose A37, batch FF1837, Genneviliers, France). Berol EMU-043 was obtained from MoDoKemi, AB (Stenungssund, Sweden). Triton X-100 (scintillation grade) was purchased from Serva (Heidelberg).

Complement lysis. The membranes of sheep erythrocytes lysed with an excess of antibody and complement were prepared as in ref. 11 and washed four times with ice-cold 5 mM phosphate, pH 8.0 (10 min at $25\,000 \times g$ in a Sorvall RC-2B centrifuge, rotor SS 34).

Membrane solubilisation. The concentrated membrane suspension obtained after the last wash in 5 mM phosphate (protein concentration 8–9 mg/ml) was solubilized by addition of a 20 % (v/v) aqueous solution of Triton X-100 to a final Triton concentration of 1 %. These samples were directly utilized in quantitative rocket immunoelectrophoresis experiments, and could also be directly used for immuno-double diffusion. In experiments utilizing crossed and crossed-line immuno-electrophoresis, the samples were centrifuged at $9 \cdot 10^6 \times g \cdot$ min in a Spinco ultra-centrifuge (Model L2-653, rotor type SW 50.1, 0.8 ml adaptor) and the clear super-natant fluids utilized.

Solubilization with Berol EMU-043 was performed as in ref. 14.

Immuno-double diffusion and immunoelectrophoresis. The agarose and buffer system was as previously described [14–16]. All gels contained 1 % (v/v) Triton X-100 or 1 % (w/v) Berol EMU-043. Both detergents yielded identical results. Double diffusion, quantitative rocket immunoelectrophoresis and crossed immunoelectrophoresis with intermediate gel techniques were performed as described in refs 20–24.

Extraction procedures. In all cases, the concentrated membrane suspensions obtained after the last 5 mM phosphate wash were directly utilized. The following procedures were used in extraction experiments: (1) dialysis against 1 mM EDTA, pH 8.0 at 4 °C for 36 h [25–27]; (2) dialysis against 100 mM EDTA, pH 8.0, at 4 °C for 36 h: (3) dialysis against 1.2 M NaCl plus or minus 5 mM EDTA, pH 8.0, at 4 °C for 36 h [28]; (4) serial dialysis against the above three solutions at 4 °C for a period of 24 h against each buffer; (5) extraction with isotonic salt solutions at 37 °C over 3 h as in ref. 12; (6) repeated heating of membranes to 45 °C in 5 mM phosphate for periods of 90 min; (7) treatment of membranes with 1 mM *p*-chloromercuribenzoate sulfonate as in ref. 29.

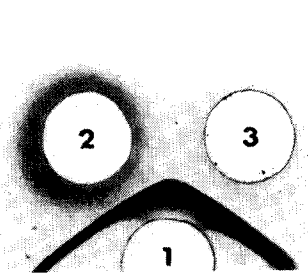
After dialysis, the ghost suspensions were centrifuged at $1.2 \cdot 10^6 \times g \cdot$ min. The clear supernatant fluids were aspirated and stored at −20 °C. The membrane pellets were re-suspended exactly to their original suspension volumes and used either in quantitative rocket immunoelectrophoresis or dialysed against the next buffer system.

For rocket immunoelectrophoresis, all samples were then made 1 % (v/v) in Triton X-100 and directly applied to the sample wells.

RESULTS

Analysis of membrane-bound C3

Double-diffusion experiments where anti-C3, serum, and solubilized target membranes are applied to different wells, yield strong precipitates with membrane-bound C3; these coalesce entirely with native C3 (Fig. 1). The membrane-bound C3



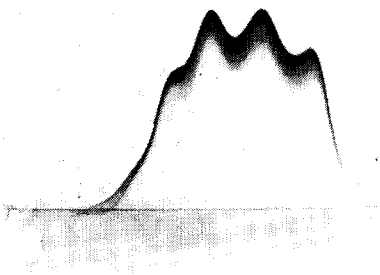


Fig. 1. Immuno-double diffusion in agarose containing 1 % Triton X-100. 1, 10 μl anti-C3 (β1c and β1a) antibodies; 2, 10 μl complement-lysed membranes, solubilized in 1 % Triton X-100; 3, 10 μl human serum. Staining: Coomassie brilliant blue.

Fig. 2. The precipitation pattern of Berol-solubilized, membrane-bound C3 component in crossed immunoelectrophoresis. 20 μl membrane material applied; the agarose contained 1.3 μl anti-C3 antibodies/cm^2. Electrophoresis conditions as in ref. 14.

component also precipitates well in crossed immunoelectrophoresis, where it resolves as a set of fusing peaks (Fig. 2), as described previously [14].

The migration velocity of these precipitates lies between the β1a and β1c regions, where native (serum) C3 and converted C3 (C3b) have their positions (30). Desorption experiments, where membrane-bound C3 is eluted into the fluid phase after incubating membranes in isotonic salt solutions at 37 °C [12], show that the eluted molecule resolves as a homogeneous entity whose migration velocity corresponds to β1a (Fig. 3C). After partial desorption the precipitation pattern of remaining, membrane-bound C3 shows that all peaks are diminished (Fig. 3). Two explanations for these findings are possible. First, the multiple peaks could represent

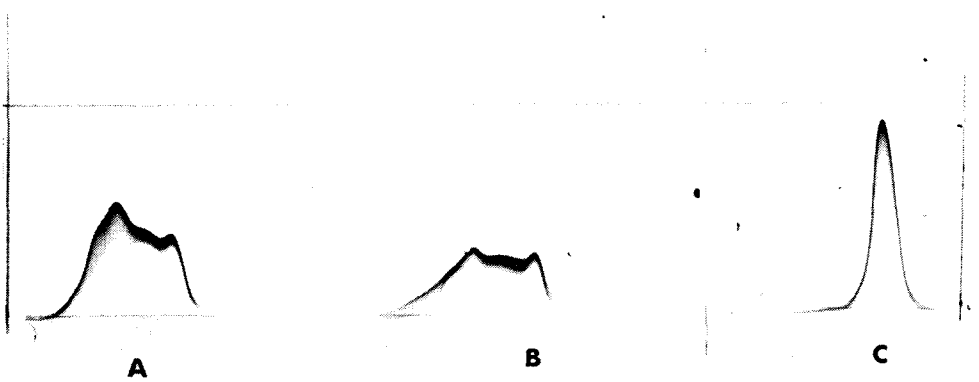


Fig. 3. Crossed immunoelectrophoresis of Berol-solubilized, membrane-bound C3 before (A) and after (B) desorption by incubation of membranes in isotonic buffer at 37 °C for 3 h. Note diminishing of all peaks after C3 elution. The desorbed C3 component resolves as a single, homogeneous entity (C). Conditions as in Fig. 2.

intermediates between native C3 and converted C3 (C3b) present on target membranes; then conversion would be a prerequisite for desorption at 37 °C. Alternatively, the multiple peaks could reflect complexing between activated, membrane-bound C3b and other membrane constituents, i.e. membrane proteins. Dissociation from these "receptors" at 37 °C would then yield the symmetrical C3b precipitate which we observe in the eluate.

Analysis of membrane-bound C5

During activation and attachment to target membranes, C5 is cleaved into a large fragment, C5b, that becomes membrane bound, and a smaller fragment, C5a, that remains in the fluid phase and possesses biological activities [31, 32]. The C5 component which we detect must therefore represent C5b or its derivative.

In double diffusion, we observe good precipitating properties of this component with anti-C5 (Fig. 4A). The precipitate fuses totally with the precipitate of native (serum) C5. Often, a second, faint precipitate is observed, which also coalesces with the serum C5 precipitate.

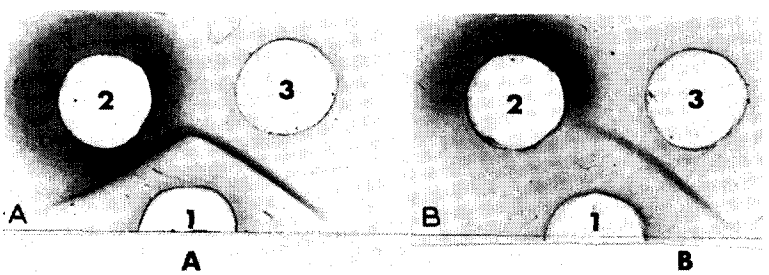


Fig. 4. Precipitation of membrane-bound C5 (A) and C6 (B) complement components in double diffusion. 1, 20 μl monospecific anti-C5 (A) and anti-C6 (B) antisera; 2, 25 μl Triton-solubilized complement-treated membranes; 3, 15 μl fresh human serum. 1 % Triton X-100 present in all gels and samples. Staining: Coomassie brilliant blue.

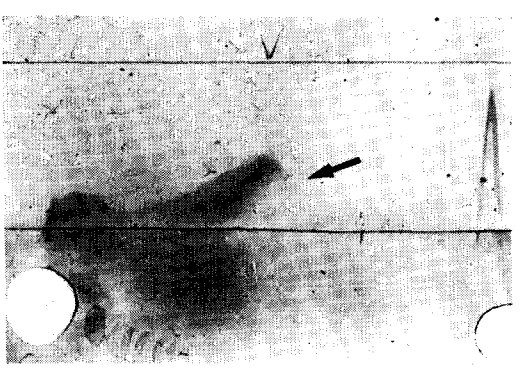


Fig. 5. Rocket and crossed immunoelectrophoresis of Triton-solubilized membrane-bound C5 (10 and 30 μl, respectively). Note formation of rocket precipitate (right). Blurred precipitates form in crossed immunoelectrophoresis; descending limbs of the precipitate are not discernible (arrow). First dimension electrophoresis: 10 V · cm^{-1} for 30 min; second dimension: 2 V · cm^{-1} for 16 h. Anti-C5 concentration: 10 μl/cm^2.

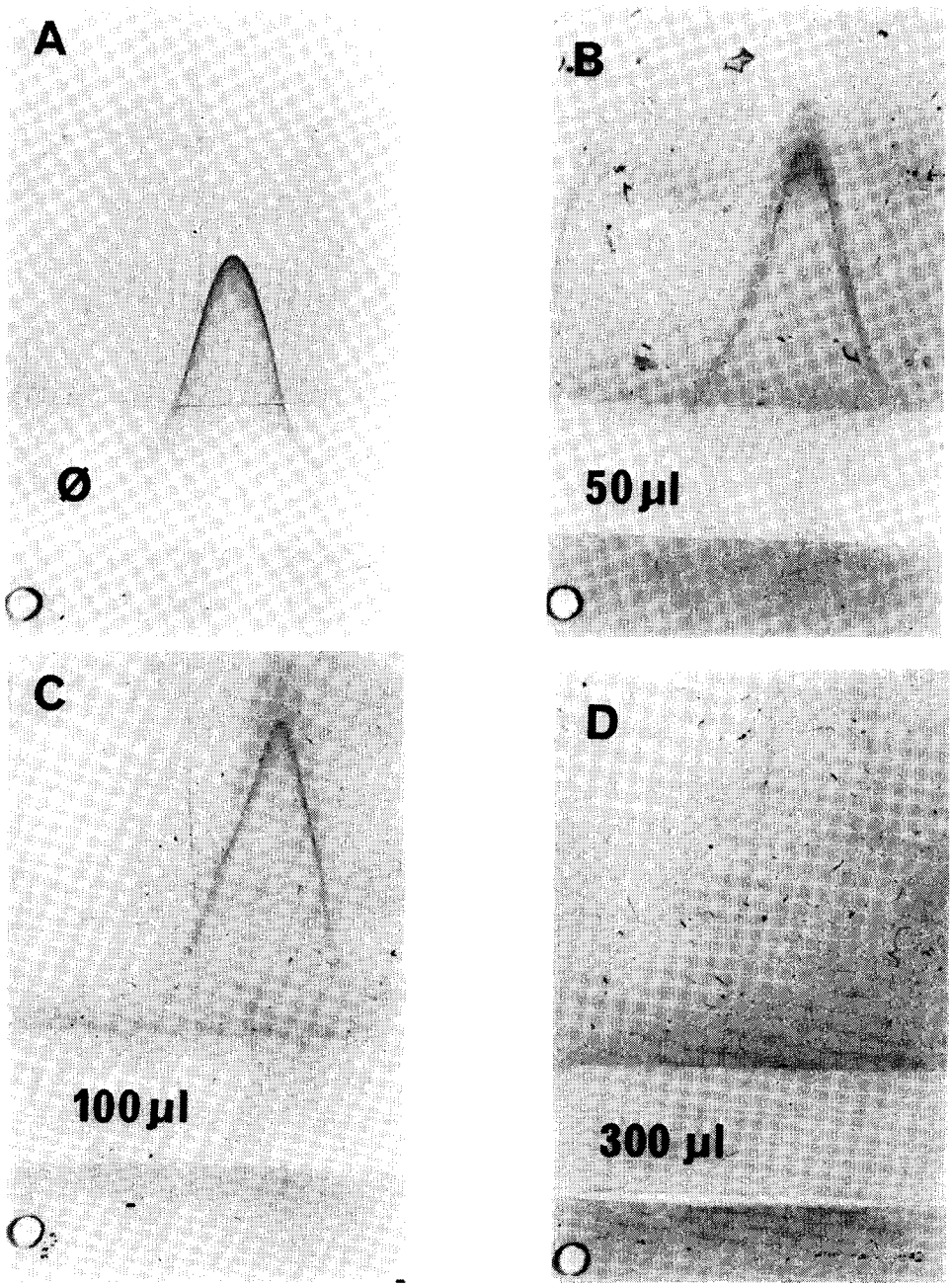


Fig. 6. Crossed-line immunoelectrophoresis utilizing in situ absorption of monospecific anti-C5 antibodies to demonstrate "loss" of antigenic components of membrane-bound C5. 5 μl of normal human serum applied in each plate. 7 μl/cm^2 anti-C5 antibodies incorporated in the reference/upper gels. A, blank intermediate gel; B, intermediate gel containing 50 μl; C, 100 μl; D, 300 μl solubilized complement-treated membranes. First dimension electrophoresis: 10 V · cm^{-1}, 45 min; second dimension: 2 V · cm^{-1} for 16 h. Note progressive uplifting of the serum C5 precipitate due to antibody absorption in the agarose gels by membrane-bound C5.

In rocket immunoelectrophoresis, membrane-bound C5 also precipitates to form clear rockets. However, crossed-immunoelectrophoresis gives blurred ascending precipitates which lack descending limbs (Fig. 5, arrow).

Basically two explanations for this phenomenon may be discussed. First, it could reflect cleavage or denaturation of the membrane-bound C5 molecule, compared to the native C5 component against which the antibodies are directed; this precipitation behaviour has been described for other proteins [33]. However, earlier studies utilizing immunoelectrophoresis indicate that cleavage of C5a from the parent C5 molecule during activation of C5 in a fluid phase does not result in a loss of antigenic determinants which would explain the precipitation behaviour observed [31]. We therefore favor a second explanation, i.e. that the precipitation pattern reflects complexing between C5 and other membrane/complement components, in analogy to the pattern obtained for plasmin- α 2-macroglobulin complexes [34]. In this case, it is believed that the precipitation pattern arises due to the presence of heterogeneous states of the molecules in the samples; these are separated electrophoretically in the first dimension electrophoresis. The antigenic determinants of the faster moving molecules are shielded by other molecules to which they are complexed and therefore reveal no precipitates (missing descending limbs, arrow in Fig. 5).

Such alteration and "loss" of antigenic determinants of membrane-bound C5 can also be directly demonstrated by crossed-line immunoelectrophoresis utilizing in situ absorption of anti-C5 antibodies [23]. In this, serum C5 is run against monospecific anti-C5 in crossed-line immunoelectrophoresis. Incorporation of a blank intermediate gel provides the control. When solubilized target membranes are incorporated into the intermediate gel, the antigenically "deficient" membrane-bound C5 molecules bind antibodies in the upper gel without forming precipitates. The complexes are therefore electrophoresed out of the gels, resulting in a reduction of antibody titer. This in turn causes the serum C5 precipitation arc to rise and also to become fainter. Fig. 6 shows that this effect is dependent on the amount of membrane material incorporated in the intermediate gels. In all these experiments, including those performed on C6 and C9 components, agarose with a congeletion point of 37 °C was used to avoid heat denaturation of complement components.

Analysis of membrane-bound C6

Complement component C6 has been analysed utilizing antibodies directed against rabbit C6 which also cross-react with human C6. We have therefore performed experiments using target membranes lysed both with rabbit and with human complement. The results are equivalent in both systems.

Available data indicate that C6 is not cleaved during assembly on target membranes [1, 6]. However, even simple Ouchterlony double diffusion shows a loss of some antigenic components of C6 in its membrane-bound state. Thus, the precipitation is extremely faint, and there may be spurring over of the native (serum) C6 precipitate (Fig. 4B). Moreover, no clear precipitates are observed in either rocket or crossed immunoelectrophoresis.

Upon "crossed-line immunoelectrophoresis" with in situ adsorption by antibodies, membrane-bound C6 behaves similarly to C5. However, the serum precipitation arcs are raised to a lesser degree, even after incorporation of large amounts of membrane material in the intermediate gel. This further indicates that C6 in its mem-

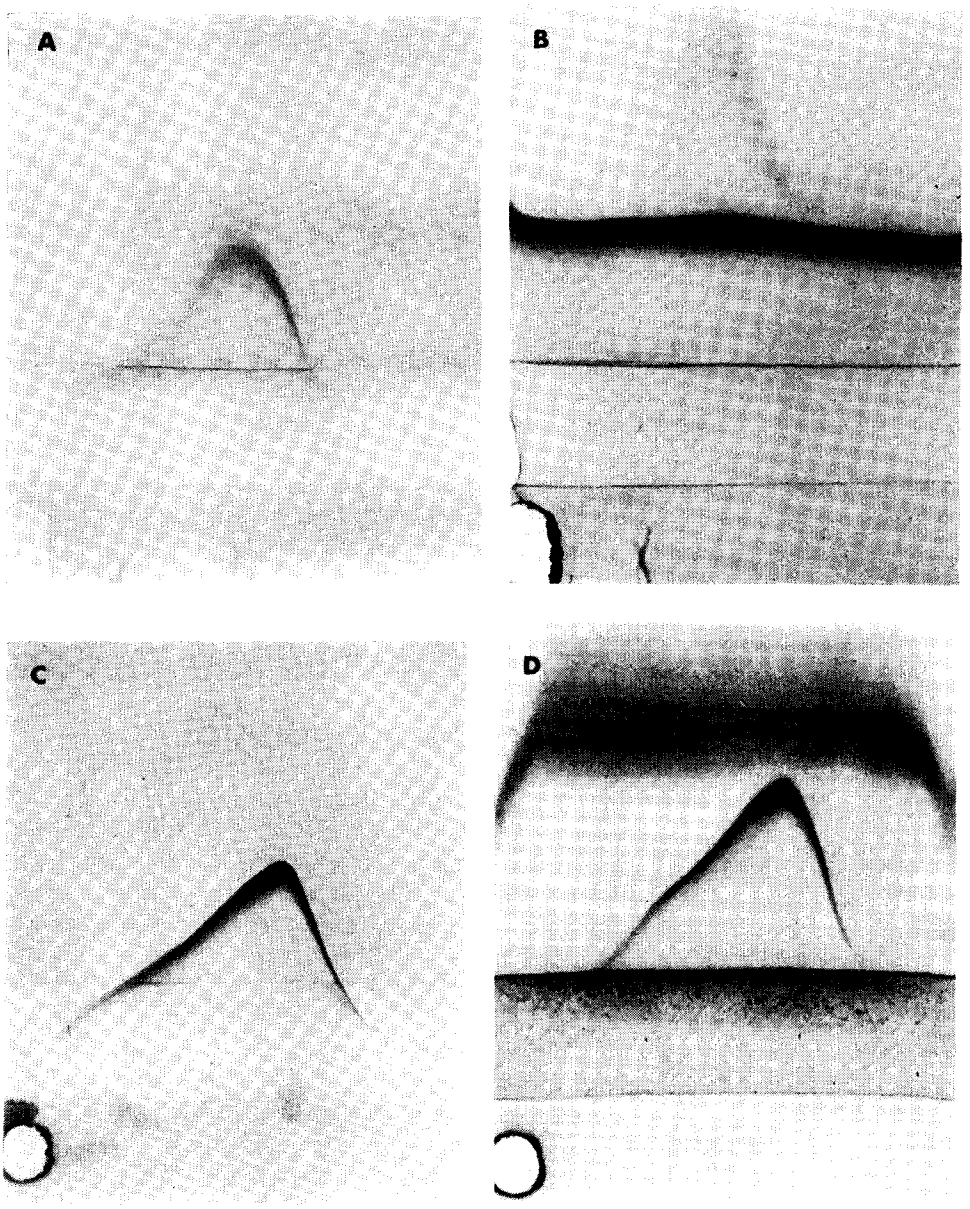


Fig. 7. Crossed immunoelectrophoresis of serum and membrane-bound C9. A, 40 μl complement-lysed membrane material (2.5 mg/ml protein), blank intermediate gel; B, as A, but intermediate gel containing 60 μl fresh human serum (15 μl/cm^2); C, 20 μl human serum, blank intermediate gel; D, as C, but intermediate gel containing 300 μl complement-lysed membrane material (2.5 mg/ml protein). First dimension electrophoresis: 45 min at 10 V $\cdot$ cm^{-1}. Anti-C9 concentration in upper reference gels: 5 μl/cm^2.

brane-bound state undergoes important structural changes, resulting in the loss of antigenic determinants.

Analysis of membrane-bound C9

C9 is the last component to react and attach to target membranes. The molecule is not cleaved during this process [1, 35]. We find that membrane-bound C9 precipitates well in double diffusion, fully coalescing with the serum C9 precipitate. The pattern is virtually identical to that found for C5, and is therefore not shown. Also, membrane-bound C9 readily precipitates both in rocket and in crossed immunoelectrophoresis. Fig. 7A gives the typical precipitation pattern obtained in the latter system. However, crossed-line immunoelectrophoresis again shows that membrane-bound C9 is not antigenically identical to serum C9. Thus, incorporation of human serum in an intermediate gel results in a line precipitate which totally lifts up the membrane C9 component (Fig. 7B). Continuity of the line precipitate below the membrane C9 arc is often observed. This is not an uncommon phenomenon in crossed-line immunoelectrophoresis and has been described and discussed for other serum proteins elsewhere [36, 37].

Conversely, however, incorporation of membrane material in intermediate gels fails to lift the serum C9 precipitate (Fig. 7C) onto the line precipitate (Fig. 7D). Here we find that a line precipitate of membrane C9 forms clearly, but that the serum C9 precipitate only becomes somewhat larger in area (35—40 %) by planimetric quantitation [38]) compared to the control pattern (Fig. 7C).

We cannot fully explain these findings at present. On the one hand, they indicate that certain "native" C9 determinants become shielded through complexing as with C5 and C6; this results in in situ absorption of C9 antibodies (area increase of the serum C9 precipitate, compare Figs 7D to 7C). On the other hand, formation of a membrane C9 line precipitate which appears to pass the serum C9 arc also suggests that activated, membrane-bound C9 possesses "new" antigenic determinants recognizable by the antibodies. This could be due to configurational changes of the molecule, not detectable by other immunochemical techniques as yet applied. Further studies are now underway to clarify these points.

Complexing between terminal complement components

It is well documented that terminal complement components can form complexes in solution; there is strong evidence that such complexes also form on target membranes during complement lysis [6, 7]. Our studies provide further evidence for the existence of such complexes on erythrocyte membranes.

As shown in Fig. 8A, when native serum is placed into the center well and antisera against various complement components into the surrounding wells, the corresponding precipitates form, with clear crossing over of unidentical entities and coalescence of two neighboring, identical precipitates. However, when complement-lysed target membranes solubilized in non-ionic detergent are introduced into the center well, all antisera tested yield clear coalescence (Fig. 8B). There is faint spurring over from C5 to C9, and from both C5 and C9 to C6. In the latter case, this may be due to the very weak C6 precipitate obtained. Spurring over from C5 to C9 may indicate that not all membrane-bound C5 is complexed to C9. On the other hand, all membrane-bound C9 appears to be complexed to C5.

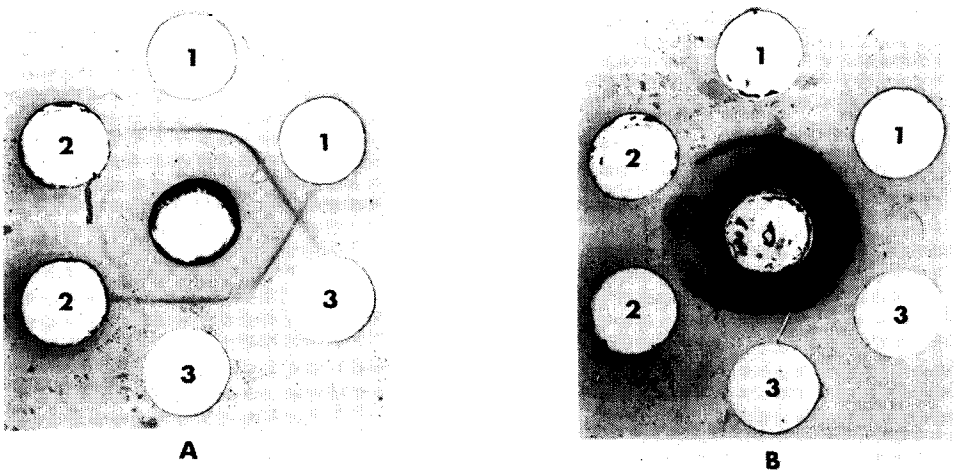


Fig. 8. (A) Analysis of terminal complement components in fresh human serum by immuno-double diffusion. 20 μl of serum applied in the center well. 1, 20 μl anti-C5 antiserum; 2, 25 μl anti-C6 antiserum; 3, 15 μl anti-C9 antiserum. Note clear coalescence of identical precipitates, but crossing over of non-identical entities. (B) Analogous experiment with application of 35 μl complement-lysed membranes solubilized in 1 % Triton X-100 (3.5 mg/ml protein concentration) in the center well. Note coalescence between all precipitates. All samples and gels contained 1 % Triton X-100. Staining: Coomassie brilliant blue.

Similar experiments utilizing anti-C3 and anti-C4 antibodies, as well as antibodies directed against sheep erythrocyte membrane proteins show that all precipitates completely cross the C5 and C9 precipitates. Thus, our system does not reveal any complex formation between early-reacting complement components and membrane proteins or between early-reacting and terminal complement components.

Extractability of membrane-bound complement components

Our data provide further experimental support for the hypothesis of Kolb and Müller-Eberhard [5–7] that terminal complement components form stable complexes with each other on target membranes. Because non-ionic detergents do not significantly dissociate the terminal complex and because complexed membrane-bound C9 readily precipitates in rocket immunoelectrophoresis, we have used this technique to evaluate the extractability of the terminal complex under various conditions. As outlined in refs 21 and 39 rocket immunoelectrophoresis allows quantitative evaluation of the data.

We first tested our approach by evaluating the extractability of membrane-bound C3 (Fig. 9A). The same sample volume was applied to all wells. Well No. 1 contained target membranes solubilized with Triton X-100. Well No. 2 contained the dilute EDTA extract of these membranes. Well No. 3 contained the pellet obtained after EDTA extraction, resuspended in 1 mM EDTA pH 8.0, to precisely the original volume. The difference in the areas delimited by precipitates 1 and 3 thus directly reflects the amount of C3 extracted by a 24-h dialysis of the target membranes against dilute EDTA. Well No. 4 contained the membranes resuspended after a further, 24 h dialysis against 100 mM EDTA, pH 8.0. The area of the rocket is virtually identical to

that of rocket No. 3, showing that this procedure does not alter the antigenic determinants of C3. Wells 5–9 contained extracts obtained after dialysis against 100 mM EDTA, pH 8.0, and subsequent dialysis against 1.2 M NaCl, and the corresponding resuspended pellets. It can be seen that each extraction procedure results in further

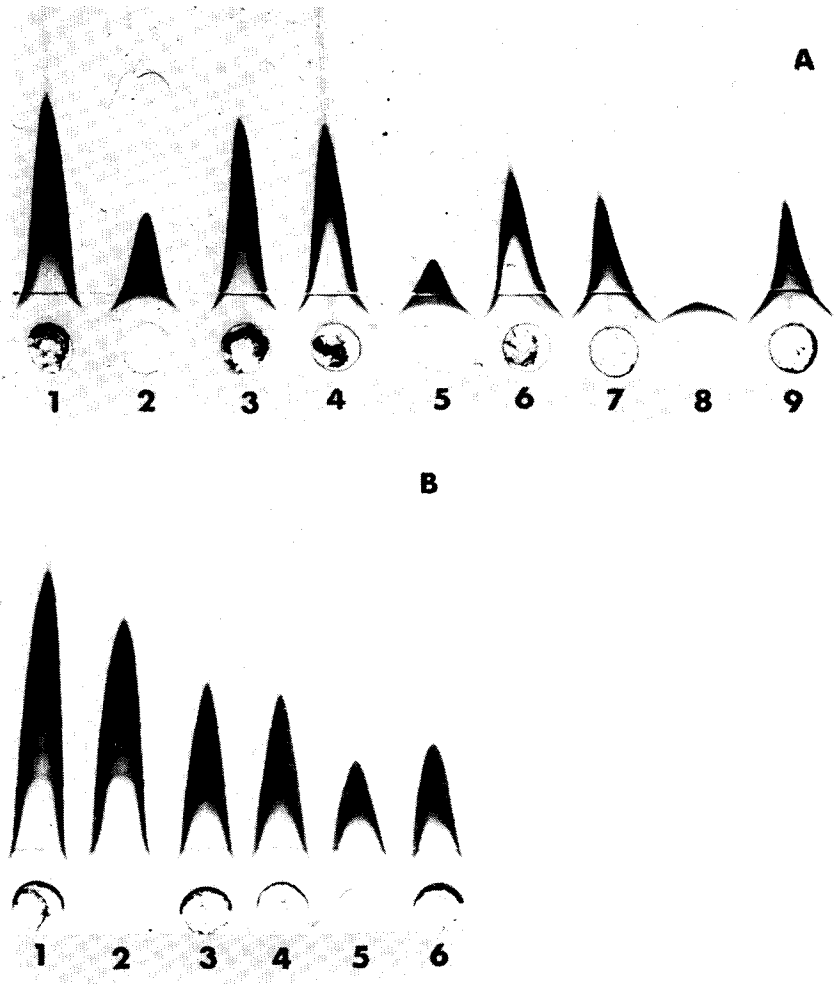


Fig. 9. (A) Desorption of C3 from target membranes by serial dialysis against three different buffers. 10-μl samples applied to each well. 5 μl/cm^2 monospecific anti-C3 antibodies incorporated in the agarose gel. All samples contained 1 % Triton X-100. 1, complement-lysed sheep erythrocyte membranes (8 mg/ml protein); 2, dilute EDTA extract of 1; 3, resuspended membranes after extraction with dilute EDTA; 4, sample 3 after 24 h at 4 °C in 100 mM EDTA, pH 8.0; 5, 100 mM EDTA extract of 4; 6, resuspended membranes after extraction with 100 mM EDTA; 7, sample 6 after 24 h at 4 °C in 1.2 M NaCl; 8, 1.2 M NaCl extract of 7; 9, resuspended membranes after extraction with 1.2 M NaCl. (B) Desorption of C3 from target membranes at 45 °C in 5 mM phosphate buffer, pH 8.0. 10-μl samples applied to each well. 1, complement-lysed membranes; 2, extract obtained after 90 min at 45 °C; 3, resuspended membrane pellet after first extraction; 4, the same membranes after further 90 min at 45 °C; 5, second extract; 6, resuspended membranes after second extraction. Rocket immunoelectrophoresis performed at 2 V . cm^{-1} for 16 h.

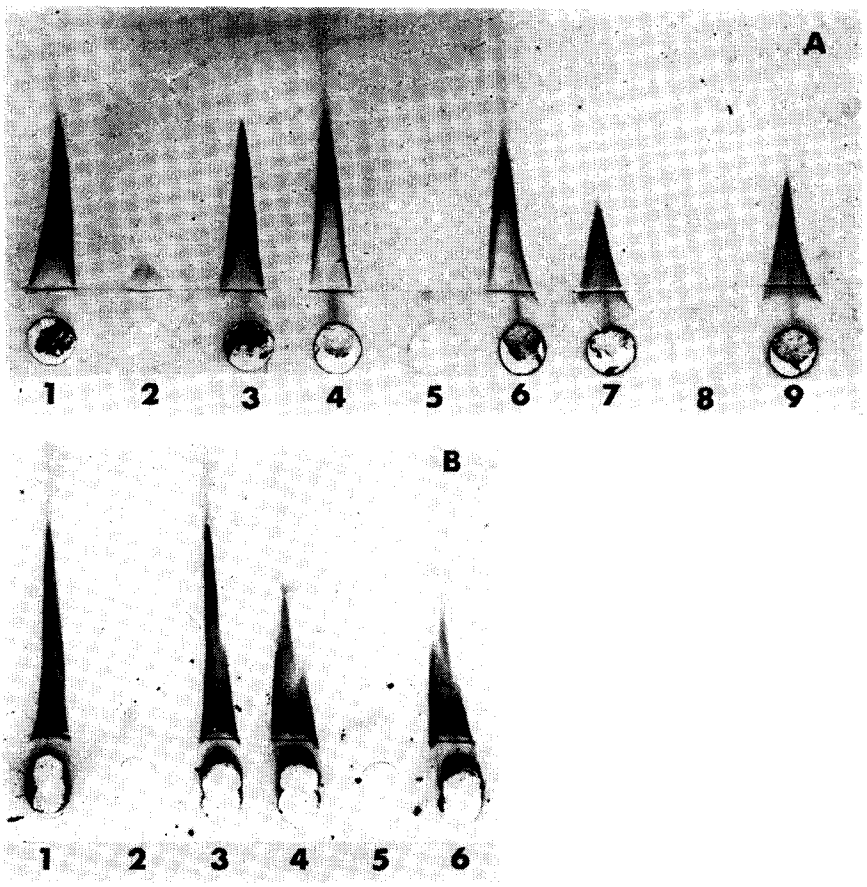


Fig. 10. (A) Desorption of C9 from target membranes by serial dialysis against different buffers. 15-μl samples applied to each well. 5 μl/cm^2 monospecific anti-C9 antisera incorporated into the agarose gel. The samples were identical to those listed in Fig. 9A. (B) Desorption of C9 from target membranes by heating at 45 °C for 2 × 90 min. 15 -μl samples applied, as in Fig. 9B. Rocket immunoelectrophoresis conditions as in Fig. 9. Note deformation of rockets after prolonged heating at 45 °C.

elution of C3 from the membranes. Dialysis against 1.2 M NaCl results in a small reduction of antigenic determinants, as shown by a comparison between rockets 6 and 7.

In another set of experiments, release of membrane-bound C3 was studied after incubation of membranes in 5 mM phosphate at 45 °C for periods of 90 min. Fig. 9B shows that under such conditions, large amounts of C3 are desorbed into the fluid phase; up to 65 % of membrane-bound C3 can be eluted by two subsequent incubations.

Figs 10A and 10B give the results of identical experiments where anti-C9 was incorporated into the agarose gels. Significantly, only minute amounts of this component can be eluted by the procedures outlined above. The good precipitation patterns obtained with the resuspended pellets shows that the absence of precipitating C9 in the eluates is not due to denaturing effects. However, after dialysis for 24 h

against hypertonic salt solutions, the C9 rocket is smaller in area, suggesting some denaturation. Moreover, prolonged incubation at 45 °C results in deformation of the C9 rocket (Fig. 10B, wells 4 and 6).

We have found that less than 6 % of the membrane-bound C9 detectable in our system can be eluted with dilute EDTA, or by any combination of elution procedures. Treatment of membranes with 1 mM p-chloromercuribenzoate sulfonate [29] also does not elute C9. Butanol extraction [40] results in the appearance of C3 but not C9 in the water phase, but we have not been able to exclude butanol denaturation of C9.

Desorption experiments carried out in isotonic buffers at 37 °C, as outlined in ref. 12 also show that, in contrast to C3 and C4, C9 is not extractable by this procedure.

Experiments performed with anti-C5 yielded similar results as with anti-C9. We have also analysed 20-fold concentrated EDTA extracts of membranes in immuno-double diffusion; this has shown that most of the C5 and C9 eluted are still in complexed form.

DISCUSSION

Our present work further documents the potency of immunological techniques in membrane protein analyses. Rather simple experimental methods provide basic information on the molecular nature of membrane-bound serum components, and the method of rocket immunoelectrophoresis of target membranes solubilized in non-ionic detergent allows quantitation of membrane-bound serum components.

The excellent precipitating properties of membrane-bound C3 with an anti-serum against the A-determinant indicates that this molecule does not undergo antigenically significant structural changes of its A-antigenicity during activation and attachment to membranes. On the other hand, crossed immunoelectrophoresis clearly shows that this component is not present in a homogeneous state, the fusing peaks suggesting that complexes exist between this complement component and other membrane constituents. The fact that desorbed C3 component gives symmetrical, bell-shaped precipitates supports this contention. Evidence for binding of C3 to membrane proteins has been previously presented in other experimental systems [11, 12]. However, the possibility that the fusing peaks represent intermediate degradation products of C3 remains to be excluded.

We examined C3 first because it is known not to directly participate in the events leading to cell lysis [1]. With the availability of monospecific antisera directed against C5, C6 and C9, we have been able to investigate complement components whose assembly on target membranes is involved in membrane lysis. These components have previously been shown to form stable complexes with each other following activation in the fluid phase [6, 7]; we have now directly documented such complexes attached to target membranes by immuno-diffusion analyses. Our studies also show that the complex is apparently stable in non-ionic detergents.

Directly related to complex formation by terminal complement components is the subsequent alteration of antigenicity. Whereas membrane-bound C5 and C9 precipitate inconspicuously in double diffusion and rocket immunoelectrophoresis, crossed and crossed-line immunoelectrophoresis show that membrane-bound C5 and C9 are antigenically deficient. These observations demonstrate the sensitivity of crossed immunoelectrophoresis techniques as such subtle molecular changes would

have escaped detection by immunodiffusion or rocket immunoelectrophoresis alone. On the other hand, loss of antigenic sites on membrane-bound C6 can be discerned by simple immuno-double diffusion. This could mean that C6 "loses" more antigenic sites than C5 and C9 during its assembly into the terminal complement complex and its attachment to target membranes. Conceivably, such loss of antigenic determinants after binding to target membranes in late-reacting complement components is due to rearrangement of the antigenic sites within the complement complex.

Erythrocyte membrane proteins can be classified operationally into two broad categories [41], i.e. "extrinsic" proteins, that can be readily eluted into aqueous media by various ionic manipulations, such as described here, and "intrinsic" proteins, that require detergents for extraction [41, 42]. In a comparative study utilizing a sensitive, two-dimensional separation system for membrane proteins, we have recently shown that dilute EDTA alone qualitatively extracts virtually all erythrocyte membrane proteins with the exception of the "intrinsic" proteins [43].

Information on diverse membrane systems indicate that "intrinsic" proteins reside at least in part within the hydrophobic membrane domains, and there is evidence that in erythrocytes, some "intrinsic" proteins span the entire membrane width [44, 45]. The fact that the terminal complement complex is almost totally refractive towards elution under conditions including ionic manipulations which extract "extrinsic" erythrocyte membrane proteins suggests that one or more terminal complement components are anchored to the membrane by penetration of peptide segments into the apolar membrane core. This possibility has been previously suggested by others [9, 46]. Conceivably, such penetration into the hydrophobic membrane interior could interfere directly with the structural equilibrium of the membrane and induce the permeability impairment responsible for complement-mediated cytolysis.

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