

Structural Polymorphism of the Fourth Component of Human Complement

STEPHEN I. ROSENFELD, SHAUN RUDDY, and K. FRANK AUSTEN

From the Department of Medicine, Harvard Medical School at the Robert B. Brigham Hospital, Boston, Massachusetts 02120

ABSTRACT The fourth component of human complement (C4) in 102 individual plasma samples has been examined by the technique of antigen-antibody crossed electrophoresis (AACE). Electrophoretic heterogeneity of C4 was manifested by the repeated occurrence of seven different precipitin patterns. These patterns were formed by varying combinations of three subtypes of C4, differing in electrophoretic mobility. The subtypes were designated C, A, and A₁, in order of increasing electrophoretic mobility toward the anode. The evidence that the observed electrophoretic heterogeneity of the C4 molecule represents structural polymorphism rests on five points: the pattern obtained from the plasma of a given individual was reproducible in different runs and with different bleedings; all seven patterns could be demonstrated on the same electrophoretic run; C4 of a given subtype retained its characteristic mobility after purification, when run alone or mixed with plasma containing C4 of other subtypes; the subtypes A₁ and C comprising pattern 6 could be separated chromatographically as well as electrophoretically; and the characteristic relative mobilities of different C4 subtypes, in plasma or after purification, were retained even after the rather large shift in mobility associated with conversion to C4i. The ratio of C4 hemolytic activity to protein concentration varied according to the subtype composition of individual samples, with highest ratios occurring with patterns composed of subtype C alone, intermediate values with patterns consisting of A and C, and lower values occurring with patterns containing subtype A alone. Although the mechanism of inheritance of this polymorphism is not yet clear, the data suggest that subtypes A and A₁ are inherited as autosomal codominant characteristics, independent of the inheritance of subtype C.

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INTRODUCTION

Genetic polymorphism has been described in many human serum proteins, including albumin, transferrin, haptoglobin, Gc globulin, immunoglobulins, α_1 -antitrypsin, beta lipoprotein (1), and most recently, the third component of complement (C3)¹ (2-4). The polymorphism of some of these proteins has been demonstrated by showing differences in electrophoretic mobility of functionally or antigenically identical proteins or by showing minor antigenic differences among the same proteins in different individuals. In the case of the light chain genetic marker of immunoglobulins, Inv, the amino acid substitution responsible for the genetic polymorphism has been described (5).

In the complement system, polymorphism has been described involving either changes in mobility or variations in quantity or functional activity of several components. Two types of polymorphism manifested by diminished function are illustrated by the disease hereditary angioedema (HAE). In some families, the affected individuals are deficient in the inhibitor of C $\bar{1}$ (C $\bar{1}$ INH) measured both functionally and immunochemically (6, 7). In other families (6), the protein is present immunochemically but lacks functional activity, implying a structural alteration of the molecule near its functional site. In the case of hereditary deficiency of the second component of complement (C2), the deficient state is transmitted as an autosomal characteristic, with heterozygotes having levels of C2 protein and function about one-half that of normals, and homozygotes having 4-5% of the normal level (8, 9). In the case of C3, Alper and coworkers have described a number of elec-

¹The nomenclature used conforms to that agreed upon by the World Health Organization (*Bull. World Health Organ.* 39: 935). E refers to sheep erythrocytes, A to rabbit antibody to E. In order of their reaction, the components are designated C1, C4, C2, C3, C5, C6, C7, C8, and C9. The activated state of a component is signified by a bar above the number, e.g., C $\bar{1}$.

trophoretic variants (3), as well as a partial deficiency state which is inherited as an autosomal dominant trait, and most likely represents nonexpression of one allele (10).

The fourth component of complement (C4) is present in serum at a concentration of approximately 400 $\mu\text{g}/\text{ml}$, about one-third that of C3. When agarose, paper, or starch-block electrophoretograms of whole serum or plasma are stained for protein, no band corresponding to C4 can be reliably identified. In order to examine the electrophoretic behavior of C4 in individual plasma samples, it was therefore necessary to adapt Laurell's technique of antigen-antibody crossed electrophoresis (AACE) (11). With this method, polymorphism of C4 is clearly apparent, and a genetic basis appears likely.

METHODS

7 ml of blood from volunteer subjects were drawn into B-D Vacutainer tubes containing 9 mg disodium ethylenediaminetetraacetate (Na_2EDTA) and promptly centrifuged at 4°C . In most cases, the plasma was frozen at -70°C within 2 hr after venipuncture; occasionally, plasma was held at -20°C for 1-2 days before being transferred to a -70°C freezer. Donors were chosen at random from laboratory personnel, but follow-up of family members was frequently based upon the finding of an unusual pattern. All subjects were American Caucasians.

Isolation and measurement of complement components

Human C4. C4 was purified from serum of individual donors according to the method described in (12). These preparations were free of the functional activity of other complement components except for minor contamination with C1INH and the ninth component. On immunoelectrophoretic analysis with anti-normal human serum, several contaminating proteins, including albumin, IgM, IgG, and occasionally ceruloplasmin were detectable, usually as faint lines.

Stoichiometric hemolytic titrations of C4 in serum, plasma, or purified preparations were performed by a modification of the method of Ruddy and Austen (12). Functionally pure guinea pig C2 was employed in place of human C2. For the titration of plasma samples, the dextrose-gelatin-Veronal buffer, pH 7.5, (DGVB⁺⁺) used for the initial dilution contained 0.00115 M Ca^{++} rather than the 0.00015 M Ca^{++} used for serum titrations; the concentration of Mg^{++} (0.0005 mole/liter) was the same for serum and plasma titrations. Under these circumstances, titrations of serum and plasma obtained simultaneously from the same individuals showed no differences in C4 titer. C4 protein measurements in both serum and plasma were performed by radial immunodiffusion (13).

Functional measurements of C4 after separation by agarose electrophoresis were performed by cutting 2-mm wide strips longitudinally from the area occupied by the β -globulins. 2- to 3-mm sections of these strips were crushed into 0.05 ml of DGVB⁺⁺ (containing 0.00115 M Ca^{++}) in microtiter plates. These eluates were serially diluted in DGVB⁺⁺ using calibrated dilution loops and tested with the same reagents as used for the stoichiometric titrations. Strips parallel to those cut for elution were used in AACE to compare the functional activity with the protein precipitation pattern.

C4 in purified preparations was converted to hemolytically inactive C4i by incubating it with 0.05 U of C1 per unit of C4 for 10 min at 30°C . Conversion of C4 in whole serum was performed by incubating it with 1 mg zymosan (Mann Research Labs.) per ml of serum for 30 min at 37°C .

Human C1 . Human C1 was prepared as described in reference 7, and measured by the method of Borsos and Rapp (14).

Preparation of antiserum to C4

Two rabbit antisera to human C4 were used in this study. One rabbit (C4-1) received, over a 5 month period, 2.3×10^9 EAC4 (15) in Freund's complete adjuvant administered in the toe pads, followed by 110 μg of partially purified C4 from pooled human serum (12) injected intravenously, followed by C4-anti-C4 precipitin arcs cut from a washed immunoelectrophoresis plate and administered in Freund's complete adjuvant into the popliteal lymph nodes of the rabbit. The resultant antiserum, obtained 2-4 wk after the final injection, was rendered monospecific by absorption with 4 mg/ml of lyophilized serum from a patient with HAE (having less than 50 μg of C4/ml) and with 20 mg/ml of Cohn fraction II. The absorbed antiserum gave a single line on immunoelectrophoresis and Ouchterlony analysis against normal human serum and against partially purified C4 from this laboratory as well as highly purified C4 kindly supplied by Dr. Hans Müller-Eberhard. The arc produced by antiserum C4-1 in immunoelectrophoresis against normal human serum was identical in position to and fused with the arc produced by a specific antiserum to C4 kindly supplied by Dr. Müller-Eberhard.

The second rabbit (C4-4) received, over a 3 month period, one dose of C4-anti-C4 precipitin arcs administered in Freund's complete adjuvant into the popliteal lymph nodes, and two intravenous doses of 100 μg of partially purified C4 prepared from pooled human plasma by the method of Müller-Eberhard and Biro (16) supplied by Hyland Laboratories. This antiserum, obtained 2-4 wk after the final injection, reacted with normal serum to give a strong precipitin arc with C4, as well as weaker lines with IgM, IgG, and IgA. This serum was rendered monospecific by absorption with a euglobulin preparation from the serum of a patient with Waldenström's macroglobulinemia. Its specificity for C4 was demonstrated by the same criteria used for C4-1. Both antisera gave only a single ring when used for radial immunodiffusion measurements of C4 in whole serum or plasma, at antibody dilutions of 1:10 (C4-1) and 1:25 (C4-4) in agar.

Antigen-antibody crossed electrophoresis (AACE)

The method of Laurell was modified, using barbital buffer, pH 8.9 (4°C), ionic strength 0.04, containing 0.002 M EDTA. The apparatus initially used was patterned after that designed by Laurell (11), and was obtained from Metaloglass Inc., Roxbury, Mass. Subsequently, to achieve a longer separation, a new apparatus was constructed which had twice the distance between electrode wicks.

Initial separations were carried out on plates of 16 oz glass 11×20 cm or 21×20 cm in a 0.7 mm thick film of 1% agarose (Fisher) in electrophoresis buffer. The plates were connected to the electrode troughs by vertical wicks and horizontal bridges of 1.5% Seravac Agarose (Gallard-Schlesinger Chemical Mfg. Corp., L. I., N. Y.) in electrophoresis buffer. The apparatus was cooled with ethylene

glycol at 2°–6°C, and potentials of about 20–22 v/cm were used, with controlled current circuitry to reduce ohmic heating. Short runs were carried out until the albumin bands (marked with Bromphenol blue) (3) had moved about 6 cm from the origin (about 45 min). Long runs were continued until hemoglobin A (3), placed in the two outer sample troughs, had migrated about 9 cm from the origin, and the albumin was in the anodal wick (about 4 hr).

For the antibody precipitation, plates similar to those used in the short run but without sample slots were poured with 1% agarose containing antiserum C4-1 at a 1:12 or antiserum C4-4 at a 1:30 dilution. 2-mm wide strips, 3.7 cm long, containing the separated beta globulins, were cut from the initial separation plate, starting from the hemoglobin region and extending towards the origin. These strips were then carefully lifted and placed in a 2 mm wide trough cut across the base of the antibody-containing agarose film. A potential of about 15 v/cm was applied for 1 hr at an angle of 90° to the direction of the previous electrophoretic separation. The antibody-containing plate was then covered with a piece of Whatman 1 filter paper moistened in saline, four pieces of blotting paper, and a glass pane; bottles of sand were then placed on the glass pane for 10 min to flatten the agarose. The antibody-containing plate was washed overnight in saline, rinsed in running tap water for 30 min, dried, and stained with Naphthol Blue Black. The initial separation plate was fixed in acetic acid-methanol-water for 10 min, rinsed for 1 hr, dried, and stained with Naphthol Blue Black.

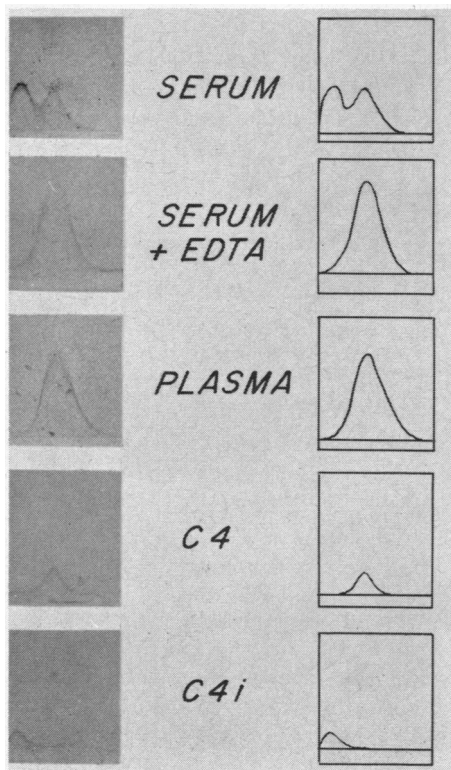


FIGURE 1 AACE analysis for C4 employing short-run technique (see text). Anode is to the left.

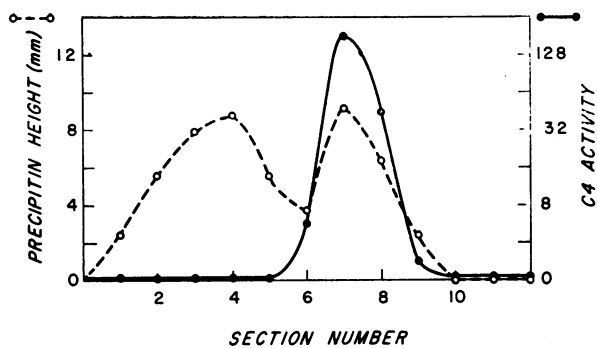


FIGURE 2 Analysis of C4 hemolytic activity (●—●) eluted from one of a pair of agarose strips obtained after the initial short electrophoretic run; the strip was cut into 2-mm sections. The other strip was analyzed en bloc by AACE; ○---○ represents height above the baseline of the C4 precipitin pattern corresponding to the sections eluted from the first strip. Anode is to the left.

RESULTS

Partial conversion of C4 to C4i during electrophoresis of serum. When AACE was performed on plasma samples using a short initial separation, only single C4 peaks were observed; but if serum samples were used, double peaks were frequently seen. When EDTA (0.005 mole/liter final concentration) was added to the serum before electrophoresis, only one peak was produced, which was in the same position as that seen in plasma (Fig. 1). Addition of 0.005 M CaCl₂ to the EDTA-containing serum before electrophoresis reversed the EDTA effect. When the protein was eluted from the initial separation and tested for C4 hemolytic activity, it was found that the more anodal of the two peaks formed from serum had no hemolytic activity (Fig. 2). If the C1 was removed from serum by precipitation at ionic strength 0.04 and pH 7.5 (17), the resultant pseudoglobulin fraction produced only the cathodal, hemolytically active C4 peak on AACE. C4i produced by treating purified C4 with C₁ migrated in the same position as the inactive anodal peak from serum (Fig. 1). Accordingly, EDTA plasma samples were used for all electrophoretic studies.

Electrophoretic characteristics of C4 in individual plasmas. When individual plasmas were studied by AACE using short initial electrophoretic separations, only single peaks of slightly variable shape were seen. Among 102 plasmas examined by AACE using prolonged initial separations, seven patterns were seen repeatedly (Fig. 3). Despite minor differences between individual runs, the pattern produced by the plasma of a given individual from the same or different bleedings was reproducible from run to run; patterns were not influenced by freezing and thawing up to five times.

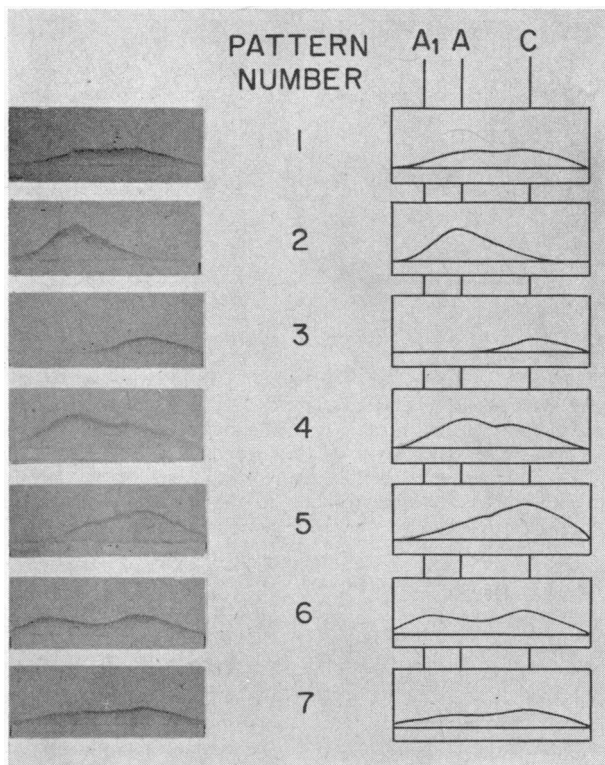


FIGURE 3 AACE using long initial separation, illustrating the seven patterns observed among individual plasma samples. The subtypes of C4 responsible for the various patterns are noted in relation to the tracings on the right. Anode is to the left.

Pattern 1, seen 27 times, has two closely placed peaks of equal height, termed C4 subtypes A and C. Both peaks A and C could be seen easily in plasmas of pattern 1 even when their C4 concentrations were as low as 180 $\mu\text{g}/\text{ml}$, which is more than two standard deviations below the normal mean (13). Four examples of pattern 2, containing only the more anodal subtype A, have been seen. Pattern 3, containing subtype C alone, has been seen in only three subjects. Pattern 4, representing both subtypes A and C, but with a clear preponderance of A, was noted in nine plasmas; pattern 5, containing more C than A, was seen in 17 samples. Patterns in which differences between the heights of the two peaks were not striking and which were intermediate to patterns 1 and 4 or 1 and 5 proved difficult to classify; six plasmas were intermediate between patterns 1 and 4, and 13 were intermediate between 1 and 5. It was never difficult to distinguish between patterns 4 and 5. If, on several runs, an individual sample showed a minor but consistent difference in the heights of the two peaks, it was classified into the appropriate asymmetric pattern. Pattern 6, containing subtype C and a subtype more anodal than A, called A₁, presents a wide separation between the two peaks; such a pattern was seen in two unrelated individuals. Pattern 7, which contains some material migrating as far anodally as subtype A₁ but with less clear separation between the two peaks, was seen in 21 relatives of the two subjects with pattern 6. Although the area under the precipitin curve of all patterns could be used as an approximate index of the C4 protein con-

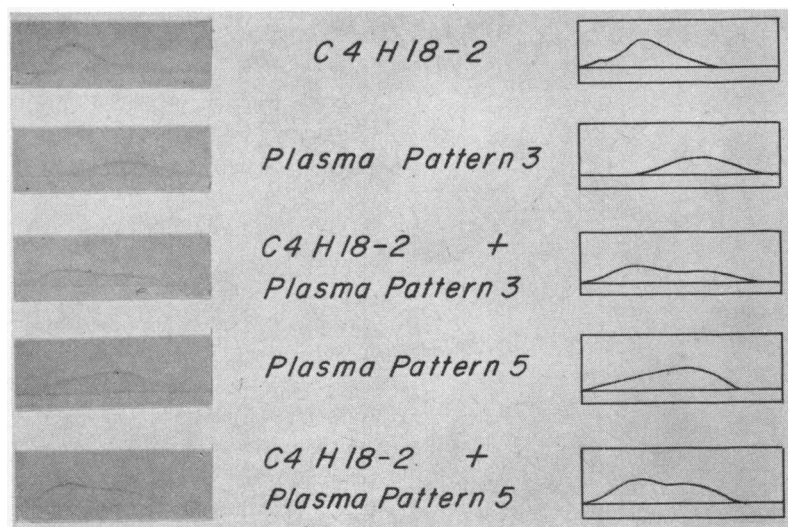


FIGURE 4 AACE analysis of isolated C4 of subtype A run in the presence or absence of plasmas giving patterns 3 or 5. Conditions as for Fig. 3.

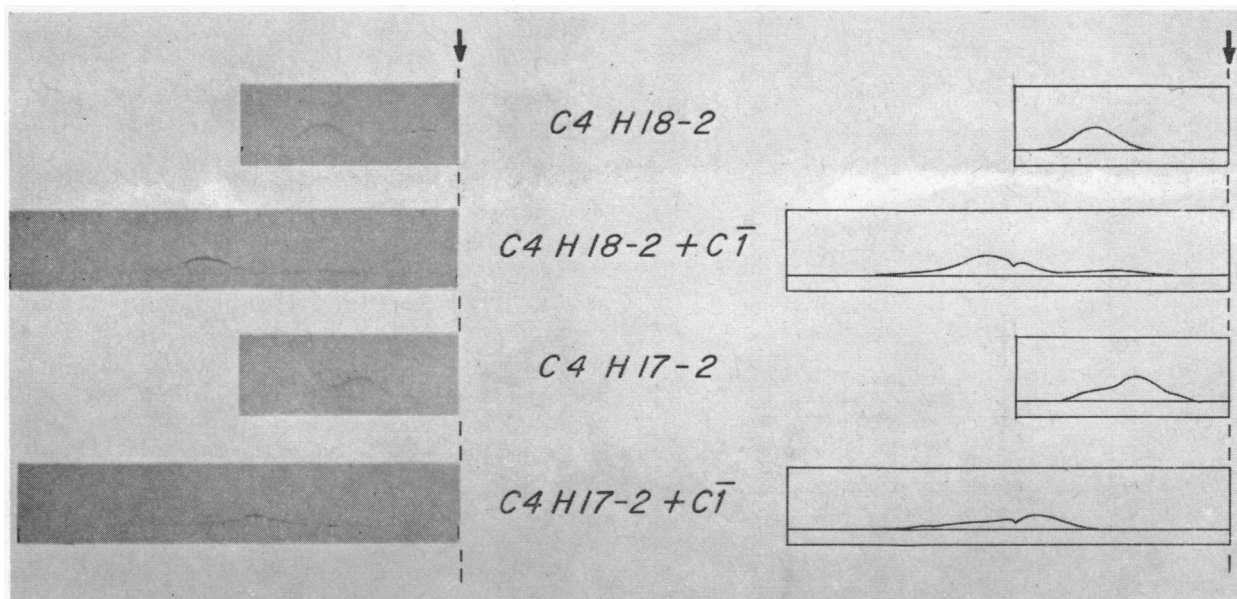


FIGURE 5 AACE analysis of C4 of subtypes A (C4H18-2) and C (C4H17-2) before and after treatment with $C1\bar{}$. Arrow indicates a distance of 4.6 cm from the origin. Conditions as for Fig. 3.

centration, the semiquantitative techniques used for sample application and for transferring gel from the initial electrophoresis plate to the antibody containing plate precluded accurate estimation of C4 concentration in starting samples from this parameter.

Persistence of electrophoretic characteristics after isolation of C4. To help differentiate between polymorphism intrinsic to the C4 molecule and that of a hypothetical protein in plasma to which C4 binds, C4 (preparation C4H18-2) was isolated from the serum of the subject whose pattern 2 is shown in Fig. 3. When this partially purified protein was run on AACE against anti-whole human serum, the only precipitate seen in the

region cut was that of C4. AACE analysis with anti-C4 revealed that this preparation had the same mobility as it had in the original plasma. The addition of this C4 of subtype A to plasmas of patterns 3 and 5 did not change its anodal mobility as analyzed by AACE; the C4 in the carrier plasmas migrated in their original cathodal positions (Fig. 4). With the antiserum used (C4-1) there was no spurring between the precipitates of C4H18-2 and the C4 in the carrier plasmas, indicating their antigenic identity. When the pseudoglobulin fraction from the serum of a subject having subtypes A₁ and C (pattern 6) was fractionated on a column of microgranular DEAE-cellulose (DE-52, Whatman) at pH

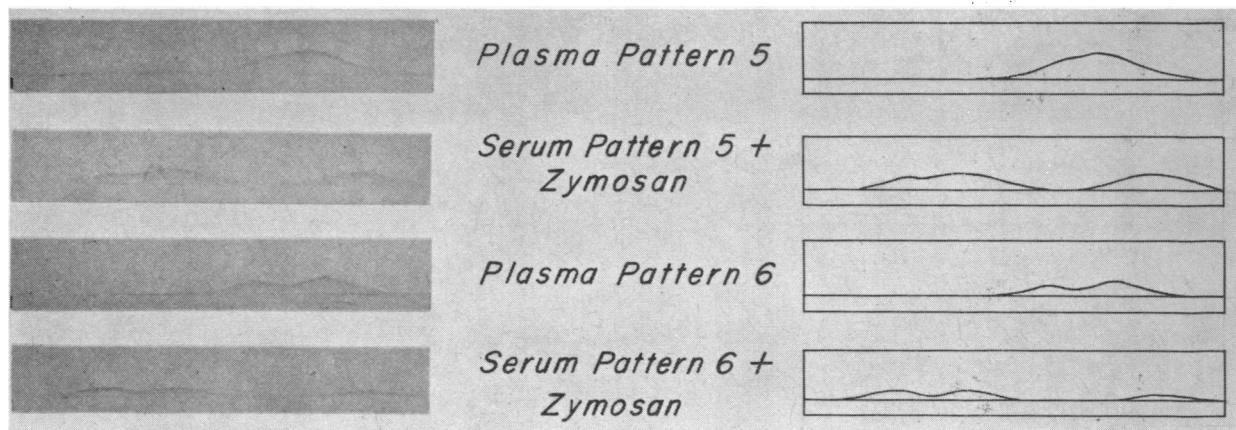


FIGURE 6 AACE analysis of patterns 5 and 6 before and after treatment with zymosan. The reason for the cathodal shift of the residual C4 is unknown. Conditions as for Fig. 3.

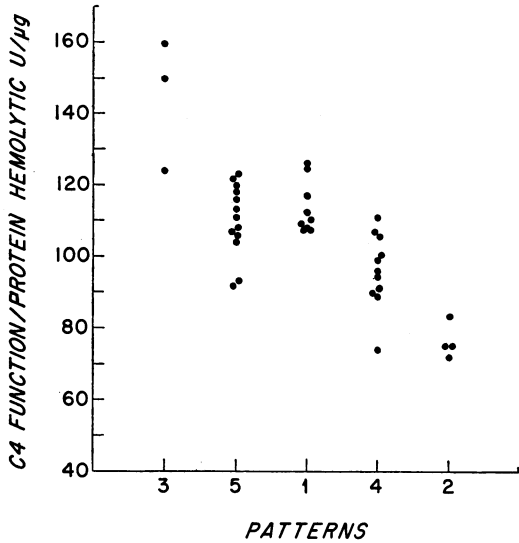


FIGURE 7 Relationship of the specific functional activity of plasma C4 to the pattern obtained by AACE.

7.5, the C4-containing fractions eluted at ionic strengths below 0.135 contained almost exclusively C4 of subtype C, and those fractions eluted at ionic strengths above

0.143 contained mostly subtype A₁, with a small amount of subtype C.

Preservation of electrophoretic differences after conversion to C_{4i}. A purified C4 preparation (C4H17-2), isolated from the serum of a donor of type 1 before any knowledge of the electrophoretic heterogeneity was available, contained C4 which was predominantly of subtype C, presumably because of the manner in which fractions from the original DEAE-cellulose column were pooled. This preparation was easily distinguished from C4H18-2 on AACE. When each of these purified C4 preparations was treated with C₁I, the resultant C_{4i} preparations migrated much faster than the original C4, and maintained the same relative differences in mobility as had been observed with the untreated C4 preparations (Fig. 5).

When serums from subjects of types 1 and 6 were incubated with zymosan, partial conversion of their C4 to C_{4i} occurred. It can be seen in Fig. 6 that the two peaks present in each serum became converted, and that the relative differences in their mobilities were preserved.

Functional studies of C4 subtypes. Among 40 plasmas thus far studied, the ratio of C4 hemolytic activity to C4 protein concentration appears to be lower in those sam-

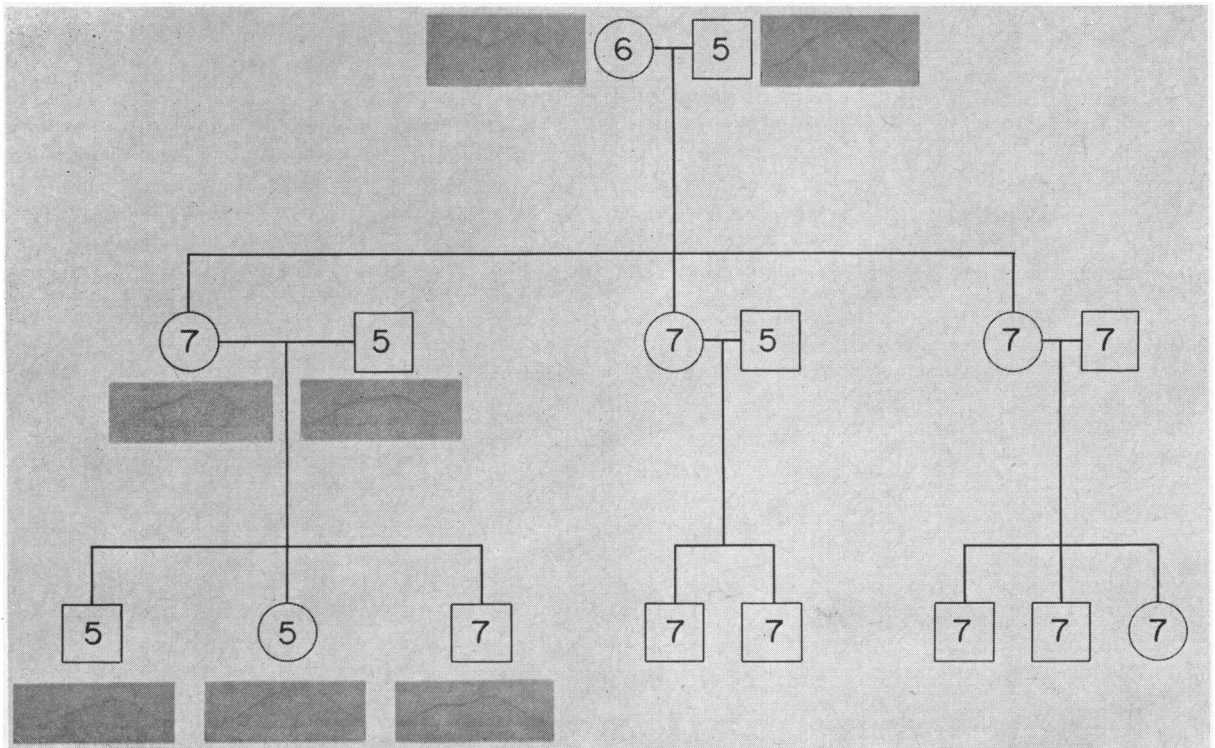


FIGURE 8 Gol family. Numbers within the circles (females) and squares (males) refer to C4 patterns on AACE (see Fig. 3).

ples containing completely or predominantly C4 of subtype A (patterns 2 and 4) than in those samples with slower moving C4 (pattern 3); plasmas of patterns 1 and 5 have intermediate values (Fig. 7); two individuals with pattern 6 had values of 73 and 84 U/ μ g; these values are lower than those observed in individuals with pattern 1, implying that the substitution of subtype A₁ for A is associated with a reduction in specific functional activity. Since it has not yet proved possible to prepare purified C4 with 100% of the activity of the same protein in whole serum or plasma, verification of this trend by analysis of purified preparations is not available. Elution of C4 from the agarose strips is not sufficiently quantitative to detect small differences in the activity of separate peaks reliably, but reveals that all three subtypes have hemolytic activity.

Family studies. The results of matings so far studied are shown in Table I. C4 migrating in the anodal region A₁ (patterns 6 and 7) has been found in only two kindreds so far (families Gol and Gra), and in one of these families (Gol) has been found in each of the three generations studied (Fig. 8). All of the offspring of the two individuals of type 6 had patterns containing at least some C4 of subtype A₁ (pattern 7), and in each case it appeared to replace part of the A peak rather than the C peak.

Another informative mating was that between two individuals of pattern 5, which yielded three offspring, two of pattern 5 and one of pattern 3 (Fig. 9). The only other individual of pattern 3 for whom genetic data were available was the product of a mating between individuals of patterns 1 and 5 (Fig. 10). From Table I

TABLE I
Numbers and Patterns of Offspring from
Observed Matings*

Patterns of parents	No. of matings	No. of offspring having patterns listed						
		1	2	3	4	5	6	7
1 × 1	3	8	0	0	2†	3‡	0	0
1 × 4	1	1	1	0	1	0	0	0
1 × 5	6	5	0	1	0	8	0	0
1 × 7	2	0	0	0	0	1	0	4
2 × 5	1	1	0	0	1	0	0	0
4 × 4	3	1	2	0	2	0	0	0
4 × 5	1	2	0	0	0	0	0	0
5 × 5	1	0	0	1	0	2	0	0
5 × 6	2	0	0	0	0	0	0	9
5 × 7	1	0	0	0	0	0	0	2
7 × 7	2	1	0	0	0	0	0	4

* Some individuals appear both as parents and as offspring.

† Both of these patterns were intermediate between 1 and 4.

‡ All three of these patterns were intermediate between 1 and 5.

|| Seven of these patterns were intermediate between 1 and 5.

it can be seen that each of the individuals with symmetrically unimodal C4 patterns (patterns 2 and 3) had at least one parent with the correspondingly asymmetrically skewed pattern (patterns 4 and 5, respectively).

DISCUSSION

The evidence that the observed electrophoretic heterogeneity of the C4 molecule represents structural polymorphism rests on five points: the pattern obtained from

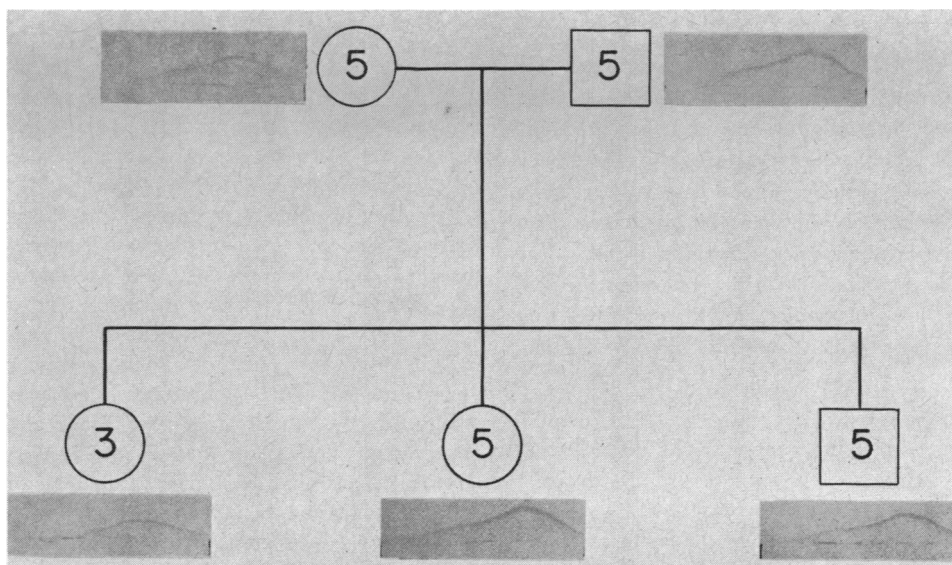


FIGURE 9 Lor family. Symbols as for Fig. 8.

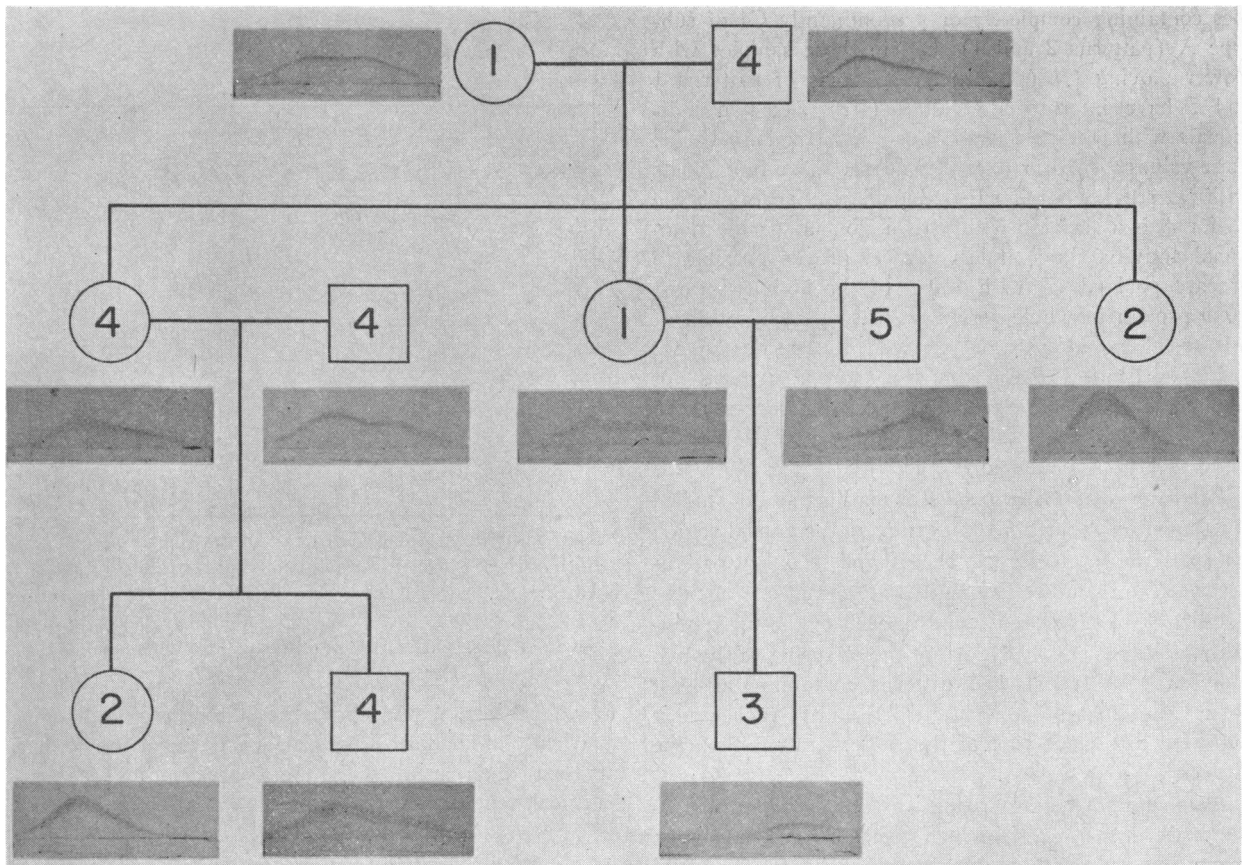


FIGURE 10 Lun family. Symbols as for Fig. 8.

the plasma of a given individual was reproducible in different runs and with different bleedings; all seven patterns could be demonstrated on the same electrophoretic run (Fig. 3); C4 of a given subtype retained its characteristic mobility after purification, when run alone or mixed with plasma containing C4 of other subtypes (Fig. 4): the subtypes A₁ and C comprising pattern 6 could be separated chromatographically as well as electrophoretically; and the characteristic relative mobilities of different C4 subtypes, in plasma or after purification, were retained even after the rather large shift in mobility associated with conversion to C4i (Figs. 5 and 6). This last point also demonstrates that the structural feature which accounts for the heterogeneity is not altered by the action of C₁I on C4. Patrick and Lepow (18) have demonstrated that the fragments of C4 produced by its interaction with C₁I dissociate at acid pH but apparently remain together on gel filtration and ultracentrifugation at alkaline pH. This association, however, apparently does not prevent the change in electrophoretic mobility associated with conversion of C4 to C4i; accordingly, it is not possible to state with certainty

which of the C₁I-cleaved fragments of C4 contains the structural heterogeneity, but in all likelihood, it resides in the larger fragment.

Cann and Goad (19) have described the phenomena of electrophoretic heterogeneity of a single macromolecule resulting from reversible binding with buffer constituents, isomerization, or interaction with other macromolecules. That the electrophoretic heterogeneity of C4 does not result from these phenomena is supported by two points previously discussed: the fact that multiple patterns could be seen on the same run with obviously identical buffer conditions, and the retention of the characteristic mobility of a given C4 after isolation and remixture with C4 of a different mobility.

The observation that C4 is frequently partially converted to C4i during agarose electrophoresis is not surprising, despite the fact that Alper and Propp (3) observed no conversion of C3 in fresh serum run on agarose electrophoresis even with calcium-containing buffers. That the hemolytically inactive, more anodal C4 peak produced by short electrophoresis of fresh serum (Fig. 1) actually represents the conversion product C4i is indicated by its electrophoretic mobility,

which is identical with C4i produced by the action of C $\bar{1}$ on purified C4. Further support for this view comes from the findings that the hemolytically inactive anodal peak fails to appear when C1 is removed by precipitation of euglobulins before electrophoresis (17) or when its activation is prevented by the presence of EDTA (20). The reasons for the failure of C3 to convert to C3i under the same conditions that frequently produce conversion of C4 to C4i are probably related to differences in the rates of these reactions. The extremely rapid rate at which C4 is converted to C4i by even small amounts of C $\bar{1}$ (21) may enable this reaction to take place before electrophoretic separation of the reactants occurs. The formation of the C $\bar{4}2$ complex necessary to cleave C3 in the hemolytic reaction sequence requires Mg⁺⁺ (22), and it is possible that the Mg⁺⁺ present in serum may be removed electrophoretically before this activation can occur.

The evidence regarding difference in functional efficiency among the subtypes of C4 is noteworthy. Alper and Propp observed no differences in functional activity of the allotypes of C3 (3), but molecular titrations were not done. The fact that the inactive conversion products, C4i, of electrophoretically different C4 subtypes also manifested distinct mobilities suggests that the differences in the mobilities of the starting materials do not represent varying degrees of inactivation. If the latter were the case, it might be expected that total conversion of the different C4 subtypes would yield identical rather than unique mobilities. One consideration yet to be ruled out involves the possibility that the structural change giving rise to the electrophoretic heterogeneity might affect the protein estimation by radial immunodiffusion. If, in fact, the functional differences observed are related to the structural changes responsible for the electrophoretic variability, one would suppose from the relatively small changes in function that the amino acid substitutions are not at the active site.

The mode of inheritance of the polymorphism of C4 appears to be more complex than the multiple allelic systems reported for C3 (3, 4) or transferrin (23). In the C3 system, a single locus, presumably controlling the synthesis of one of the peptide chains of C3, accounts for all the polymorphism so far observed. The partial deficiency of C3 in the family reported by Alper, Propp, Klemperer, and Rosen (10) appeared to be controlled by an allele at the same locus. A similar situation appears to exist for the polymorphism and deficiency of serum α_1 -antitrypsin (24). There are insufficient family studies at this time to permit firm conclusions as to the inheritance of the C4 polymorphism described. Nonetheless, the data that are available are consistent with the interpretation that subtypes A and A₁ are controlled by allelic genes, and that these genes are not allelic with that

controlling subtype C. Each of these loci appears to be autosomal in location. The supposition that A and A₁ are governed by allelic genes is based entirely on the observation that whenever subtype A₁ appears (families Gol and Gra) it apparently replaces part of the A peak, but never the C peak (Fig. 8). Individuals having pattern 7 could be considered heterozygous for genes controlling A and A₁, whereas pattern 6 individuals would be homozygous for the gene leading to A₁. The supposition that these genes are not alleles of the gene controlling production of subtype C is based upon the preceding point, as well as the relative rarity of unimodal patterns 2 and 3, containing only subtypes A and C, respectively, compared to the frequency of the bimodal patterns 1, 4, and 5, containing both subtypes. This line of thought has led to the working hypothesis that C4 contains some peptide chains unique to each individual subtype A and C, and some chains common to all subtypes. A₁ could then have arisen from a mutation in the gene controlling the peptide unique for A. Individuals with C4 of pattern 5 could carry, on one of their chromosomes, any of several conceivable types of genes reducing the quantity of the peptide unique to subtype A, while those with pattern 3 would be homozygous for this deficiency. A similar explanation could be invoked to account for patterns 4 and 2. Persons having pattern 1 could either have a normal gene for each unique chain on each chromosome, or carry one normal gene and one defective gene for each unique chain. Confirmation of this hypothesis or the development of a meaningful alternative hypothesis will require more genetic data, as well as further knowledge of the chemical structure of C4. The large size of the C4 molecule which has a molecular weight of 230,000 and an S_{20,w} of 10S (25) is consistent with the hypothesis that it consists of more than one peptide chain, but the exact number and size of the chains are unknown.

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