Evidence for Complement (C4) on Electrophoresis of Serum Fractions in Acid-Urea Starch-Gels[1] (36005)

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(Introduced by R. F. Schilling)
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Genetically controlled polymorphism of a number of human serum proteins, including the third component of complement (C3) (1–3) has been described. We were interestd in studying polymorphism of other complement proteins; however, direct identification of these proteins on electrophoretic analysis of serum proved to be impractical due to their low serum concentration and the complexity of the protein pattern of serum. In the course of exploring various methods of analyzing trace serum proteins for genetic studies, we noted one method which gave discrete bands, several of which appeared to be related to the complement system. The methodology employed, the evidence that three of the bands are C4 protein, and the results of a genetic analysis are reported here.

Materials and Methods. Fractionation of serum was achieved by addition of methanol to serum under acid conditions at 23°, an adaptation of an older procedure for separation of serum (4). One milliliter of serum; 0.5 ml of ammonium acetate/acetic acid buffer, 0.012 *M*, (pH 3.6); and 3.5 ml of 60% methanol were mixed and incubated at 23° for 15 min. The mixture was centrifuged at 46,000*g* for 30 min and the precipitate was washed with 40 ml of distilled water by resuspending and grinding in a Potter-Elvehjem homogenizer. After centrifugation the precipitate was resuspended in 2 ml of water, shell frozen, and lyophilized. The dried samples were extracted for 1 hr in a Soxhlet apparatus containing a constant boiling mixture of 85% chloroform and 15 % methanol, following which the samples were lyophilized and stored at −20°. Washing of the initial precipitate with water served to reduce contaminating γ-globulin, while the lipid extraction step reduced distortion of the patterns obtained on subsequent electrophoresis. For electophoresis, 3 mg samples, representing on the average 0.5 ml of serum, were solubilized in 0.1 ml of electrophoresis buffer containing 8 *M* urea. The samples, after homogenization and centrifugation at 46,000g for 15 min, were electrophoresed in vertical starch gels at constant current (55–60 mA and 3.9 V/cm starting voltage) for 30 hrs. The starch gels consisted of 70 g of electrostarch,[3] 300 ml of 0.03 *M* barium lactate buffer (pH 2.2–2.5), and 240 g of urea (8 *M*). To achieve optimal resolution of proteins, the buffer concentration in the gel may have to be altered, depending on the particular lot of starch that is used (5). The gels were stained with 1% amido black in 2% acetic acid. In some experiments C3 allotypes were determined by high-voltage alkaline starch-gel electrophoresis (1, 2).

The C3, C4, and C5 were purified by published techniques (6–8) and labeled with ^{125}I by the chloramine-T method (9). Biologically inactive unlabeled C4 (10) was obtained through the courtesy of H. Haupt of the Behringwerke, Marburg-Lahn, Germany.[4] These preparations were added to certain

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[4] The preparation of C4 was crystallized three times. Analysis by immunoelectrophoresis with antiserum to human serum gave one line, and polyacrylamide gel analysis revealed a single band. Hemolytic activity was lost because of the ammonium sulfate used in the fractionation (10).

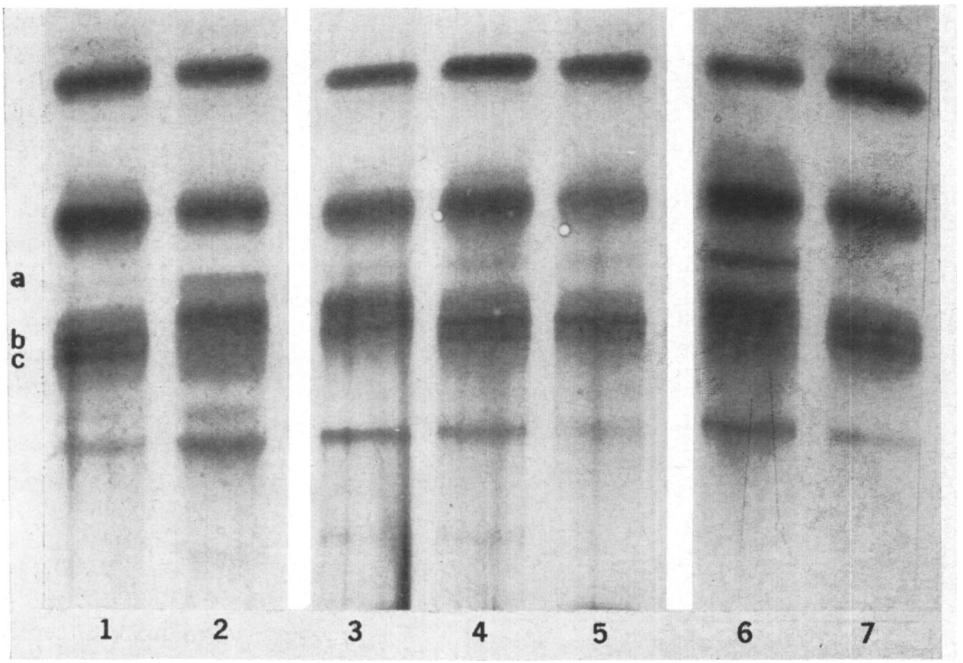

FIG. 1. Evidence of C4 proteins in acid-urea starch gel: bands (b) and (c) probably represent C4 proteins and (a) is a conversion product of C4. (2) Serum exposed to antigen–antibody complex precipitate at 4° for 16 hr; (1) incubated control for serum exposed to antigen–antibody complex precipitate; (6) serum treated with 0.03 *M* NH_4OH at 37° for 1.5 hr; (7) incubated control for serum treated with NH_4OH; (3–5) representative patterns of C4 proteins in 3 fresh serums; (3) band (b) present; (4) band (b) = (c); and (5) band (b)>(c).

serum samples prior to processing and electrophoresis. After electrophoresis, the gels were sectioned and the radioactivity present in the slices determined. Other treatments of serum employed were absorption with γG-globulin isolated from anti-C4 antiserum[5] in the presence of EDTA, and treatment with 0.03 *M* ammonia or 0.01 *M* hydrazine for 30 min at 37°. Serum samples were also treated overnight at 4° with an immune precipitate.

Fresh serums were collected at random from 143 individuals for population studies and 65 serum samples from 14 Caucasian families were obtained for further genetic study. The population data were analyzed according to the Hardy-Weinberg equation (11). Statistical analyses were performed by the chi square method.

Results and Discussion. Figure 1 (channels 3, 4, 5) shows the pattern obtained with fresh human serum. Two closely spaced bands, termed b and c, were found in most normal sera, although their relative intensities in these sera differed. A more rapidly migrating band, termed a, was usually present. It was noted early that bands b and c were absent from aged serum samples and from serum samples incubated at 37° for 12 hr. In both instances, intensification of band a was evident. These observations suggested that the proteins represented by bands b and c were related to the complement system and that band a might be a conversion product of one or both of these proteins. This hypothesis was supported in the following studies.

Serum samples were treated prior to separation with hydrazine or with ammonia,

[5] Gamma globulin purified from Hyland Co., anti-C4 serum by DEAE-cellulose chromatography (Dr. Harold Deutsch, Dept. of Physiological Chemistry, Univ. of Wisconsin). The anti-C4 serum was found to be monospecific by testing in immunoelectrophoresis.

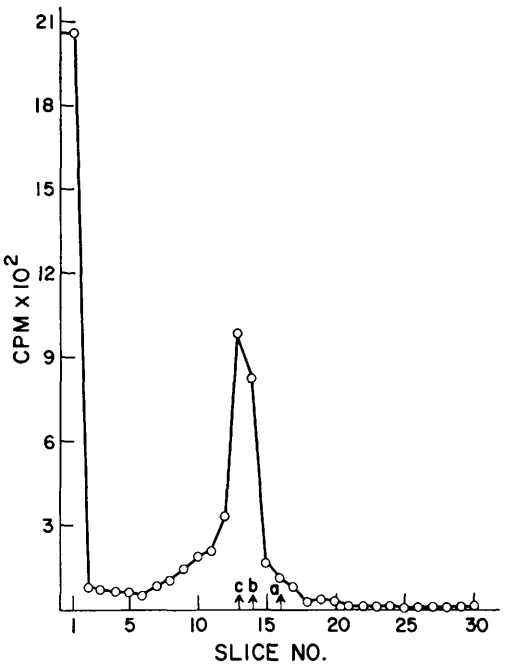

FIG. 2. Biologically active, radiolabeled ^{125}I-C4 was added to serum which was processed and electrophoresed in acid-urea starch gel. The gel was sliced and the slices counted for radioactivity. Arrows at the bottom indicate the position of the bands in question (a, b, and c) and suggest (b) and (c) as representing unconverted C4.

chemicals which alter the physiocochemical properties of the third and fourth components of complement (12). This treatment resulted in diminution or disappearance of bands b and c and intensification of band a (Fig. 1, channel 6). The intensity of staining of the area between bands a and b on the starch gel was also increased. In other experiments, serum samples were incubated prior to separation with an immune complex, a treatment which results in physiochemical changes of several of the complement components, especially C3 and C4, as assessed by immunoelectrophoretic analysis in agar with specific antisera (12). Acid-urea starch-gel electrophoresis of such serum samples revealed loss of bands b and c and intensification of staining of band a and the region between bands a and b (Fig. 1, channel 2). The changes in immune complex-treated serum were prevented by incorporating 1×10^{-2} *M* EDTA in the serum prior to treatment with the immune complex.

The above results were compatible with identification of bands b and c as C3, C4, or possibly C5, and band a as a conversion product of one of these proteins. Unfortunately, it was not possible to directly analyze eluates from the starch gels with antisera to the complement components, since antigenicity was lost in the acid-urea system. Several observations, however, suggested that bands b and c did not represent C3. The protein band patterns did not correspond to the C3 allotype bands of the same serum samples. For example, serums with single-banded C3 2-2 or double-banded C3 1-2 phenotypes determined by high-voltage alkaline starch-gel analysis gave similar double-banded patterns (bands b and c) in acid-urea starch-gel separations. Studies with serum samples to which ^{125}I-C3 had been added prior to separation showed a broad peak of radioactivity throughout the area encompassed by bands a, b, and c, but no discrete peak over any of these bands. The distribution of radioactivity on analysis of serum to which ^{125}I-C5 had been added was also diffuse throughout the area in question.

Next considered was C4, and the following studies suggest that bands c and probably b are related to C4 and band a is probably a conversion product of C4. Purified radiolabeled biologically active C4 was added to serum prior to separation.[6] Although a major portion of the radioactivity remained at the origin, a discrete peak of radioactivity was also located over the position of bands b and c (Fig. 2). A smaller amount of radioactivity above base line value was located in the position of band a. The reason for the radioactivity at the origin is unknown, but it may represent aggregated C4. In other experiments, purified, unlabeled, biologically inactive C4 was added to serum before processing.[4] Under these conditions, band a was intensified in proportion to the amount of C4

[6] The radiolabeled C4 was analyzed by immunoelectrophoresis with antiserum to human serum, giving a single line. Also polyacrylamide gel analysis gave a single band. Radiolabeling the C4 did not result in loss of hemolytic activity (7).

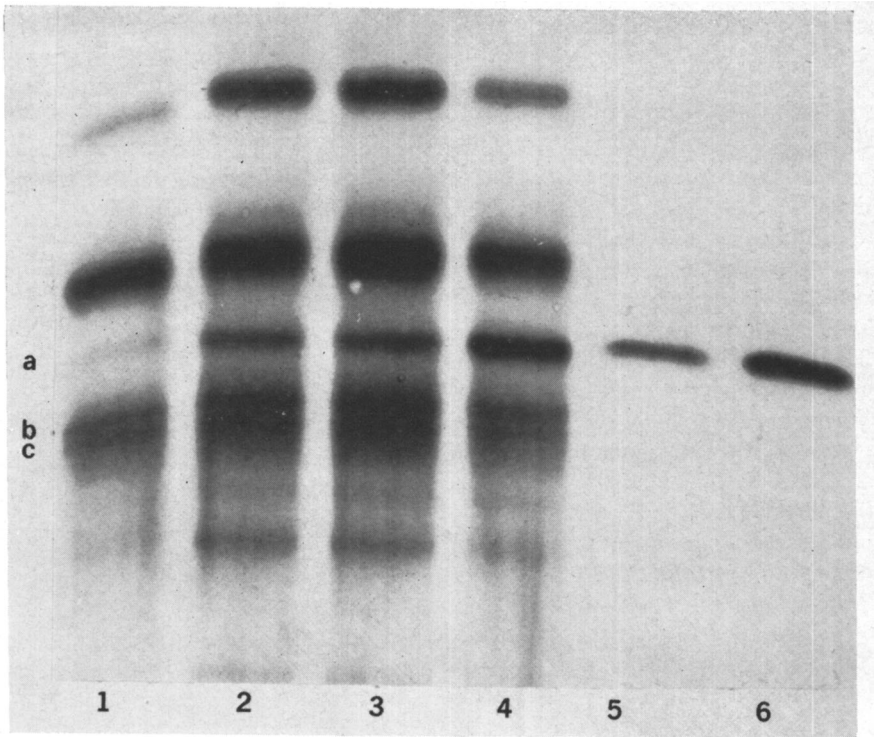

FIG. 3. Unlabeled, biologically inactive, purified C4 electrophoresed alone or added to serum which was processed and electrophoresed in acid-urea starch gel. Evidence is offered that band a is converted C4. (1) Serum; (2, 3) serum + C4 (75 mg %); (4) serum + C4 (300 mg %); (5) C4 (75 mg %); and (6) C4 (150 mg %).

added (Fig. 3, channels 2, 3, 4). The same C4, processed in the absence of serum, gave a single band identical in mobility to band a (Fig. 3, channels 5 and 6). Additional evidence that bands a and c and probably b are related to C4 was obtained by depleting serum of its content of C4 by absorption with the γ-globulin fraction of anti-C4.[5] After removal of the precipitate, the serum samples were processed. This treatment resulted in removal of bands a and c. This may also have been true for band b, but the added γ-globulin overlay the region of band b.

Randomly collected fresh sera were studied in an attempt to establish a simple inheritance pattern for the "C4 proteins." Bands b and c varied in intensity in these serum samples from different individuals. These differences were reproducible on repeated study; the relative intensity of the bands was not altered by prolonged incubation of the samples. For the purpose of testing autosomal dominant inheritance at a "C4 locus," C4 patterns, in which band b was present alone or greater in intensity than band c, were postulated to represent one homozygous pattern (Fig. 1, channels 3 and 5). The heterozygous pattern was considered to be present when b and c were of equal intensity (Fig. 1, channel 4). The alternative homozygotic pattern was postulated to be represented by a greater intensity of band c relative to band b. Of 108 random, freshly collected sera from Caucasian individuals, there were 70 postulated homozygotes, 38 postulated heterozygotes and 0 postulated alternative homozygotes. The expected numbers, assuming autosomal codominant inheritance, would be 72.5, 31.9, and 3.6, respectively. In 35 serums from Negroes, 19 postulated homozygotes (20.7 expected), 16 postulated heterozygotes (12.4 expected) and 0 postulated alternative homozygotes (1.9 expected) were found. These values were in

agreement with the postulated codominant pattern at a "C4 locus" ($p = .17$). However, a study of 65 family members, including 37 children of 14 families, did not confirm this pattern of inheritance. Although of 8 doubly homozygous matings (b>c), all 25 children were homozygotes of the same class; the results of postulated double-heterozygous and homozygous–heterozygous matings differed significantly from expectation ($p = .04$). Of three double heterozygous matings, there were 7 homozygotes (b>c) and 3 heterozygotes. Of three homozygous–heterozygous matings, there were four homozygotes (b>c) and two heterozygotes. Of great importance, there was failure to find a single serum containing the postulated alternative homozygote (c>b) in either the randomly collected serums or in the serums obtained for family studies; whereas a total of 8 homozygotes of this type was expected.

It is likely that the "C4 bands" we obtained in the present study are related to the two forms of C4 obtained by Rosenfeld *et al.* (13) using antigen–antibody crossed-gel electrophoresis. They recognized three subtypes of C4, termed C, A, and A1, in order of increasing mobility with A1 being rare in frequency; they could not explain the observed pattern on the basis of a single mode of inheritance. We were also unable to explain our genetic and family studies on the basis of autosomal codominant inheritance at a single "C4 locus." It may be, as also suggested by Rosenfeld *et al.* (13) that the different "C4 patterns" represent a more complex mechanism of inheritance.

Summary. A method of analyzing trace serum proteins is presented which involves acid methanol precipitation of serum proteins, extraction in a Soxhlet with chloroform and methanol, and electrophoresis of the redissolved proteins in acid-urea starch gels. Relatively few bands are obtained which are well separated and discrete. We have presented evidence that two of the bands represent the fourth component of complement, C4, and a more rapidly migrating band is a conversion product of C4. We were unable, after analyzing 143 randomly collected fresh sera and sera from 65 members of 14 families, to establish a pattern of autosomal codominant inheritance at a "C4 locus."

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