OCCURRENCE OF AN INCOMPLETE C8 MOLECULE IN HOMOZYGOUS C8 DEFICIENCY IN MAN*

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Homozygous complement deficiencies have been characterized by the total or near total absence of the deficient complement component. Total absence of the protein has also been reported in several individuals with homozygous C8 deficiency (1-3). Recently a second form of genetic C8 deficiency has been described in which an antigenically deficient, nonfunctional C8 molecule occurs in the plasma of the afflicted individuals (4-7). We have studied the abnormal C8 protein from three unrelated individuals with functional C8 deficiency and a history of recurrent Neisserial infections. The three proteins resembled each other in that they were immunochemically indistinguishable, lacked one of the chains present in the normal three-chain C8 molecule, and could be functionally reconstituted by providing the missing chain isolated from normal C8. Parts of this study have been presented in abstract form (6,7).

Materials and Methods

Sera and Complement Components. Plasma samples from patients R.S. and J.C., who had recurrent meningococcal meningitis, were prepared at the Center for Disease Control in Atlanta, Ga. by standard procedures. Large amounts of plasma from one individual (R.S.) were obtained by plasmaphoresis performed by Dr. Michael Frank at the National Institute of Allergy and Infectious Diseases. Plasma from the third patient (S.K.), who was suffering from gonococcal infections, was supplied by Dr. C. Casavant and Dr. R. Jacobs, University of California, School of Medicine, San Francisco. Serum samples were obtained by centrifugation of plasma allowed to clot at room temperature for 1 h after addition of CaCl₂. Normal human serum was prepared from blood of donors at Scripps Clinic and Research Foundation. Complement proteins C5b-6, C7, C8, and C9 were isolated as described (8-11). The noncovalently linked subunits of C8, C8α-γ and C8β (10) were isolated as described by Steckel et al. (12). Samples of these subunits for preliminary tests were kindly supplied by Dr. James Sodetz, Department of Chemistry, University of South Carolina at Columbus.

Hemolytic Assays. Hemolytic assays of whole serum (CH50) were performed according to standard methods (13). C8 activity was measured by adding diluted test serum to human serum immunochemically depleted of C8 and by assaying for hemolytic activity as described before (10).

Sedimentation Analysis. Ultracentrifugation studies were performed with a model E analytical ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) equipped with an optical scanner.

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and a special ultraviolet light collimator (Schrader, Feinmechanik and Optik, Braunschweig, West Germany) allowing analysis of very dilute protein samples. C8 R.S. or C8e-γ R.S. dissolved in Tris-buffered saline (TBS) \(^1\) \((10 \text{ mM Tris, } 150 \text{ mM NaCl, pH 7.4})\) at a concentration of 0.1 mg/ml were sedimented at 52,000 rpm and the sedimentation rate constants \((s_{20,w})\) were calculated according to standard procedures. Equilibrium sedimentation analysis was carried out at 12,000 rpm and the protein distribution in the cell was measured at 280 nm with the photoelectric scanner. Molecular weights were determined \((14)\) from a least-squares fit of log \(A_{280}\) vs. \(r^2\) using a partial specific volume of 0.723 ml/g as calculated from the amino acid composition listed under Results.

**Sucrose Density Gradient Ultracentrifugation.** Samples were sedimented at 4°C in linear 10-40% sucrose density gradients prepared in TBS containing 10 mM EDTA in an SW 50.1 rotor \((\text{Beckman Instruments, Inc.) for 16 h at 37,000 rpm.})\)

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).** Electrophoresis was carried out in 9-cm-long, 1.5-mm-thick slabs prepared from 10% polyacrylamide using a discontinuous multiphasic buffer system described by Jovin et al. \((15)\) \((\text{CANALCO Sage Kit; Miles Laboratories Inc., Elkhart, Ind.).})\)

**Radioiodination.** Proteins were trace-labeled with \(^{125}\text{I}\) by using a solid state system described by Fraker and Speck \((16)\) \((\text{IODOGEN, Pierce Chemical Co., Rockford, Ill.).})\) Free iodine was removed by gel filtration on 10-ml columns of Sephadex G-25.

**Amino Acid Analysis.** Analyses were performed in a model 121-M analyzer \((\text{Beckman Instruments, Inc.) coupled to an Autolab System AA computing integrator. Samples were hydrolyzed for 24 h and half-cystine was determined after oxidation with performic acid.})\)

**Immunochemical Analysis.** Double immunodiffusion in agarose, quantitative radial immunodiffusion, and immunoelectrophoresis were performed according to conventional methods. Antisera to C8 were prepared in rabbits and goats.

**Results**

**Detection of Dysfunctional C8 Protein in C8-deficient Sera.** Plasma samples from patients R.S., J.C., and S.K. showed no hemolytic activity \((\text{CH}_{50})\). Immunochemical analysis revealed an apparent C8 protein concentration of 35-44% of normal. In addition, plasma R.S. had only 44% of normal C2, whereas plasma S.K. and J.C. had reduced levels of C5 (69 and 79% of normal), and plasma S.K. was also low in Factor H (B1H) (68% of normal). All other complement components in all three sera were within the normal range. \(^2\) C8 titrations performed on sera R.S. and J.C. indicated that these sera lacked C8 hemolytic activity (Fig. 1) and that both sera therefore contained dysfunctional C8 protein. The C8 hemolytic activity of the serum of the parents of R.S. was approximately one-half the normal value.

**Antigenic Deficiency of the Dysfunctional C8 Protein.** Double immunodiffusion using an antiserum to normal C8 revealed complete antigenic identity of the dysfunctional C8 proteins in the three patients' sera and marked antigenic deficiency compared with normal C8. As shown in Fig. 2, a reaction of identity was observed between C8 R.S. and C8 J.C. and between C8 J.C. and C8 S.K. In contrast, a pattern of partial identity was formed with C8 in normal serum, with the precipitin line of normal C8 spurring heavily over that of the deficient C8 protein. Upon immunoelectrophoresis, the abnormal C8 moved faster than normal C8 (Fig. 3), indicating that the deficient C8 had a charge different from normal C8 at pH 8.6.

**Lack of the β Chain in the Abnormal C8 Molecule.** The C8-related protein present in serum R.S. was isolated using immunooaffinity techniques. In brief, serum R.S. was

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1. Abbreviations used in this paper: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

2. A complete report detailing clinical and immunogenetic studies on the families of patients R.S. and J.C. will appear elsewhere.
brought to a 10-mM concentration in EDTA and passed over a column of BioRex 70 (Bio-Rad Laboratories, Richmond, Calif.) to remove Clq as described (17). The material in the column breakthrough was then applied to an affinity column containing rabbit anti-C8 IgG coupled to CNBr-activated Sepharose 4B. The anti-C8 selected for this purpose was capable of binding both the α-γ and β subunits of normal C8. After washing with TBS containing 2 M NaCl, the column was eluted with TBS brought to a 2-M concentration in NaCl and 3 M in guanidine hydrochloride. The material was concentrated, dialyzed, and further purified by gel filtration on Sephacryl-200. Analysis of C8 R.S. by SDS-PAGE under nonreducing conditions (Fig. 4) indicated that the protein behaved like the α-γ subunit of normal C8 and that it could be dissociated by reduction into chains resembling the α and γ chains of C8. The β chain could not be detected. Evidence that the β chain was not lost during the purification procedure, for example on the BioRex 70 resin, was obtained in the following way. Serum R.S. was incubated overnight with soluble rabbit anti-C8 IgG and the immune complexes formed were precipitated with an antiserum to rabbit IgG. When the precipitated material was analyzed by SDS-PAGE after extensive washing, no band with the electrophoretic mobility of C8β could be detected. Further, when C8 from normal serum was isolated by the immunoaffinity chromatography procedure, all three chains were obtained. Using the same procedure, there was
Fig. 3. Immunoelectrophoretic representation of C8 protein in serum R.S. compared with normal C8. Immunoelectrophoresis was carried out in 1.5% agarose gel and veronal buffer at pH 8.6. Anode was to the right.

evidence of the C8α-γ subunit but not the β chain in serum from patient S.K. (data not shown).

C8 R.S. was subjected to sedimentation equilibrium analysis and a molecular weight of 86,000 was determined, whereas the molecular weight of normal C8, determined by the same method, was 151,000. Both molecules are similar in shape having frictional coefficients, f/fo, of 1.15 (Table I). The amino acid composition of C8 R.S. was not significantly different from that of normal C8α-γ (Table II) and was independent of the method of isolation.

Functional Reconstitution of the Sera Containing Dysfunctional C8 with Isolated C8β Chain. Because C8 R.S. resembled the α-γ subunit of normal C8, serum R.S. was tested for reconstitution of its hemolytic activity by isolated normal C8β. As shown in Fig. 5, addition of C8β but not C8α-γ to serum R.S. restored the total hemolytic activity to normal. C8-depleted serum, tested simultaneously, required the combined addition of the α-γ and β subunits for restoration of hemolytic activity. The hemolytic activity of sera from patients S.K. and J.C. was also restored by addition of the isolated β chain alone.

Isolated C8 R.S. was also examined for its ability to become incorporated into SC5b-9. As shown in Fig. 6, C8 R.S. does not bind to SC5b-7 unless normal β chain is added. Panel A indicates the sedimentation behavior of the SC5b-7 complex formed in C8-depleted serum upon addition of C5b-6. Panel B shows the SC5b-9 complex formed in C8-depleted serum upon addition of C5b-6 plus excess C8. When 125I-C8α-γ was added together with C5b-6 to serum R.S. and the serum was then centrifuged in a linear sucrose density gradient, the labeled protein remained near the
FIG. 4. SDS-PAGE analysis of purified C8 R.S. (tracks 1 and 4), normal C8 (tracks 2 and 5), and β chain isolated from normal C8 (tracks 3 and 6).

TABLE I

|                     | Normal C8 | C8α-γ R.S. |
|---------------------|-----------|------------|
| s20,000             | 8.2       | 5.4        |
| Molecular weight    | 151,000   | 86,000     |
| f/f₀*               | 1.15      | 1.15       |

* A degree of hydration of 0.3 gram water/gram protein was assumed.

top of the density gradient (Fig. 6 C), indicating that it did not bind to the SC5b-7 complex that was formed. In contrast, when C8β was added to serum R.S. in the presence of C5b-6, formation of the SC5b-9 complex occurred (Fig. 6 D).

Discussion

The normal C8 molecule is composed of three nonidentical polypeptide chains (10, 12). The α and γ chains are covalently linked, and the β chain is noncovalently associated with the α-γ subunit. The results of the present study strongly suggest that the abnormal C8 found in the three C8-deficient individuals is identical to the α-γ subunit of normal C8 and that the β chain is missing. This conclusion is based on the following findings. First, C8 R.S. is identical to α-γ of normal C8 with respect to molecular weight, chain composition, electrophoretic mobility in SDS gels, amino
### Table II

Comparison of the Amino Acid Composition of C8, C8α-γ, and C8α-γ R.S.

| Residue | Amino acids/1,000 residues |
|---------|---------------------------|
|         | C8 | C8α-γ | C8α-γ R.S. |
| Lys     | 55 | 64    | 62         |
| His     | 21 | 24    | 23         |
| Arg     | 64 | 56    | 50         |
| Asp     | 97 | 94    | 98         |
| Thr     | 53 | 56    | 60         |
| Ser     | 78 | 80    | 83         |
| Glu     | 114| 113   | 117        |
| Pro     | 41 | 50    | 48         |
| Gly     | 88 | 93    | 90         |
| Ala     | 63 | 71    | 73         |
| ½ Cys*  | 41 | 29    | 31         |
| Val     | 63 | 67    | 66         |
| Met     | 19 | 14    | 13         |
| Ile     | 41 | 37    | 35         |
| Leu     | 75 | 79    | 79         |
| Tyr     | 45 | 41    | 41         |
| Phe     | 39 | 32    | 31         |

*All amino acids were determined after 24 h hydrolysis time.

* Measured as sulfonic acid derivative.

Fig. 5. Restoration of hemolytic activity of serum R.S. by addition of β chain isolated from normal C8. Serum R.S. and serum immunochemically depleted of C8 were reconstituted with physiological concentrations of C8 (60 μg/ml), C8α-γ (35 μg/ml), or C8β (25 μg/ml), respectively. Hemolytic activity was assayed as described in Materials and Methods.

acid composition, and immunochemical and functional properties. Second, C8 S.K. and C8 J.C. are immunochemically identical with C8 R.S. and all three sera could be restored with respect to hemolytic activity by addition of isolated C8β. C8α-γ R.S. did not bind to EAC1-7 (6) or SC5b-7 and was hemolytically completely inactive, in agreement with recent findings of Steckel et al. (12), who have demonstrated that neither the α-γ nor the β subunit isolated from normal C8 is capable of promoting hemolysis. These investigators have further reported that the α-γ subunit of normal
C8 does not bind to SC5b-7, in contrast to the isolated β chain, which does bind SC5b-7 (18).

Our failure to detect the β chain in the patients' sera cannot be explained by its loss during the course of isolation of the dysfunctional C8, because the antibody used in the isolation procedure recognized the β chain of normal C8. The possibility cannot be excluded, however, that an immunochemically undetectable, smaller fragment of the β chain is synthesized in these individuals.

The previously reported C8 deficiencies (1–3) were characterized by an absence of detectable C8 protein in the individuals' serum. Although this fact had been established with antiserum produced in this laboratory, it was possible that the antiserum used at that time did not recognize the α-γ subunit. Retesting several of the C8 deficient sera reported by Giraldo et al. (2) with anti-C8 used in this study confirmed the earlier results. These findings suggest that two different forms of C8 deficiency occur in man and therefore raise the possibility that C8 is encoded by more than one gene.

Summary

Sera from three unrelated individuals with recurrent Neisserial infections lacked C8 hemolytic activity, but contained a protein that is antigenically related to C8. Immunochemical analysis revealed complete identity of the C8-related protein of all three sera and a marked antigenic deficiency compared with normal C8. The C8-related protein was isolated from serum by adsorption to immobilized anti-C8 IgG, elution with 3 M guanidine, and subsequent gel filtration. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, the abnormal protein resembled the α-γ subunit of normal C8 with respect to mobility and its ability to be cleaved.
upon reduction into the α and γ chains. The β chain present in normal C8 was absent. Sedimentation equilibrium analysis indicated a molecular weight of 86,000 for the abnormal C8 protein, which is identical to that of the α-γ subunit of normal C8. Amino acid analysis revealed no significant difference between the abnormal C8 and normal α-γ. Unlike normal C8, the abnormal protein did not bind to EAC1-7 or to SC5b-7; however, upon addition to the deficient serum of β chain isolated from normal C8, hemolytic activity was restored and formation of SC5b-9 occurred. We concluded that the dysfunctional C8 protein in the three individuals' serum is identical to the α-γ subunit of normal C8 and that this form of C8 deficiency is distinct from the C8 deficiencies previously reported in which the entire three-chain protein is lacking.

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