

## Two types of dysfunctional eighth component of complement (C8) molecules in C8 deficiency in man. Reconstitution of normal C8 from the mixture of two abnormal C8 molecules.

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### Research Article

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# Two Types of Dysfunctional Eighth Component of Complement (C8) Molecules in C8 Deficiency in Man

## RECONSTITUTION OF NORMAL C8 FROM THE MIXTURE OF TWO ABNORMAL C8 MOLECULES

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**ABSTRACT** Restoration of hemolytic activity was examined in sera from seven unrelated eighth component of complement (C8)-deficient subjects. The sera fell into two groups, depending on whether hemolytic activity was restored by the addition of the  $\beta$ -chain (group 1) or the  $\alpha$ - $\gamma$ -subunit (group 2) purified from normal human C8. Antigenic analysis of these sera by double-immunodiffusion using anti-human C8 confirmed previous findings of a dysfunctional C8 in the four sera of group 1 and established the presence of a different dysfunctional C8 in one of the sera of group 2 when tested at a high concentration. Further characterization of the dysfunctional C8 molecules in the two sera by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that group 1 sera were missing the  $\beta$ -subunit and group 2 sera were missing the  $\alpha$ - $\gamma$ -subunit of the C8 molecule. Sera from either of these two groups alone did not produce hemolysis in hemolytic plates containing sheep erythrocytes coated with antibody and complement components up to C7 (EAC1-7) and C9. When sera from the two groups were added to adjacent wells in the hemolytic plates, a zone of hemolysis developed between the wells. The contribution of C8 $\alpha$ - $\gamma$  from the sera of group 1 and of C8 $\beta$  from those of group 2 to the

lysis of EAC1-7 in the presence of C9 was confirmed by the inhibitory effect of specific antibodies against the two C8 subunits. In experiments in which hemolytic activity was reconstituted by mixing sera from group 1 with sera from group 2, the serum source of C8 $\beta$  (group 2) was the limiting reagent. The dysfunctional C8 molecule in this serum was able to bind to EAC1-7. Chromatographic analysis demonstrated that the generation of hemolytic activity in the mixture of the two sera resulted from the reconstitution of the C8 molecule rather than the sequential action of the two C8 subunits.

## INTRODUCTION

The eighth component of complement (C8)<sup>1,2</sup> is an unusual component of the complement system in that it has a three-chain structure organized in two subunits, C8 $\alpha$ - $\gamma$  and C8 $\beta$ , which are held together by noncovalent bonds (1). The binding site for both the cell-bound and the fluid phase trimolecular complex C567 is situated on the  $\beta$ -chain (2, 3). The two subunits can be separated by gel filtration in the presence of sodium dodecyl sulfate (SDS) and urea and then recombined to form hemolytically active C8 (4).

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<sup>1</sup> *Abbreviations used in this paper:* C8, eighth component of complement; EAC1-3b and EAC1-7, sheep erythrocytes coated with antibody and complement components up to C3b or C7; NHS, normal human serum; PAGE, polyacrylamide gel electrophoresis.

<sup>2</sup> Nomenclature for complement components conforms with that agreed upon by the World Health Organization (1968. *Bull. W.H.O.* 39: 935-938.).

The serum from some patients with C8 deficiency does not react with specific antiserum to human C8 to produce a precipitin line in immunodiffusion analysis. These sera are thought to completely lack the C8 molecule (5-8). Other patients owe their C8 deficiency to a dysfunctional C8 molecule that cross-reacts antigenically with C8 in the serum of normal individuals (9-13). Analysis of these dysfunctional C8 molecules has established that they lack the C8 $\beta$ -chain (11, 12, 14). C8 deficiency due to the selective absence of the  $\alpha$ - $\gamma$ -subunit has not been recognized previously. In this report, we present our findings that mixing the two different types of C8-deficient sera restores hemolytic activity to the mixture. Additional experiments confirm that C8-deficient serum which fails to react with a specific antiserum to C8 under standard conditions does, in fact, contain C8 $\beta$ -subunit but lacks the C8 $\alpha$ - $\gamma$ -subunit.

## METHODS

**Sera.** Sera from seven unrelated C8-deficient subjects were examined in this study. Detailed analysis carried out previously on each of these sera permitted the distinction between one group of four sera (group 1) with dysfunctional C8 (9, 11, 13) and a second group of three sera (group 2) with antigenically undetectable C8 (5, 6, 8). All the four sera of group 1 have been shown to contain abnormal C8 consisting only of the  $\alpha$ - $\gamma$ -subunit (11, 14). Normal human serum (NHS) was obtained from healthy blood donors. All sera were stored in small aliquots in liquid nitrogen.

**Complement reagents and hemolytic assay.** Using the procedure described by Steckel et al. (4), human C8 and the C8 $\alpha$ - $\gamma$ - and C8 $\beta$ -subunits were purified from 200 g of paste of Cohn fractions I, II, and III obtained from Istituto Sieroterapico Milanese, Milan, Italy. The purity of the C8 preparation was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) analysis. This analysis revealed two bands of 85,000 and 62,000 D, under nonreducing conditions, and three bands of 70,000, 62,000, and 15,000 D in the presence of a reducing agent. A similar procedure was followed to ascertain the separation of C8 into the  $\alpha$ - $\gamma$ - and  $\beta$ -subunits. Functionally purified C9 was purchased from Cordis Laboratories, Inc., Miami, FL.

The intermediate sheep erythrocytes coated with antibody and complement components up to C7 (EAC1-7) were prepared from EAC1-3b treated with one of the C8-deficient sera (D.T.) at the dilution of 1:50, as previously described (9). We have previously shown that the abnormal C8 molecule present in D.T. serum does not bind to EAC1-7 (14).

In the hemolytic assay, 50  $\mu$ l of EAC1-7 ( $1.5 \times 10^7$ ) were mixed with the C8-deficient sera in a final volume of 250  $\mu$ l and incubated at 37°C for 30 min. The extent of hemolysis was read spectrophotometrically at 415 nm. For some experiments, 20 units of Cordis C9 were added to the assay system as an external source of C9 which, in other instances, was provided by the C8-deficient sera themselves. Hemolytic plates containing 1% agarose (Behringwerke A.G., Marburg, West Germany), 1% EAC1-7, and 1,000 units of Cordis C9 were prepared as previously described (9).

Antigenic evaluation of C8-deficient and NHS was carried out by double immunodiffusion in agarose (9).

**Antiserum.** A specific antiserum directed against human C8 was produced in rabbits by repeated subcutaneous injections of highly purified C8 in complete Freund's adjuvant. This antiserum gave a single precipitin line against NHS on agarose plates and was found to inhibit specifically the hemolysis of EAC1-7 by NHS in hemolytic plates (9). The IgG fraction of this antiserum, prepared by DEAE chromatography (9), was the starting material for the preparation of antibodies against the C8 $\alpha$ - $\gamma$ - and C8 $\beta$ -subunits and the F(ab')<sub>2</sub> fragments.

**Immunoprecipitation of normal and abnormal C8.** Immune precipitates were prepared by mixing optimal proportions of the IgG fraction from the rabbit anti-human C8 antiserum and either NHS or the sera from C8-deficient individuals (D.T. and R.G.) in the presence of 10 mM EDTA. The incubation was performed at 37°C for 30 min and at 4°C for an additional 5 d.

**Preparation of F(ab')<sub>2</sub> fragments to whole C8 and to the C8 $\alpha$ - $\gamma$ -subunits.** The immune precipitates obtained from NHS and D.T. serum (group 1) were washed three times with 5 mM phosphate-buffered saline (PBS) pH 7.4 containing 10 mM EDTA (PBS-EDTA) and then treated with pepsin (Sigma Chemical Co., St. Louis, MO) at the ratio of 50:1 (wt/wt) at pH 3.2 as described by Lachmann (15). This treatment led to the formation of F(ab')<sub>2</sub> fragments of antibodies against normal C8 or C8 $\alpha$ - $\gamma$ -subunit that were precipitated by 20% Na<sub>2</sub>SO<sub>4</sub> and dialyzed against PBS. The specificity of the F(ab')<sub>2</sub> antiwhole C8 molecule and anti-C8 $\alpha$ - $\gamma$  was confirmed by the formation of a single precipitin line in double immunodiffusion against NHS or purified C8 $\alpha$ - $\gamma$ , respectively.

After removal of the immune precipitates, the supernatants from the mixtures containing either NHS (supernatant 1) or C8-deficient serum (supernatant 2) were examined for the presence of residual antibody to C8 or to its subunits. Supernatant 1 did not give a precipitin reaction when tested against the original serum, while supernatant 2 still reacted with NHS but did not recognize either the  $\alpha$ - $\gamma$ -subunit purified from normal C8 or the  $\alpha$ - $\gamma$ -subunit present in the serum containing dysfunctional C8. Supernatant 2 gave only a faint precipitin line with purified C8 $\beta$ , but it was able to effectively inhibit the hemolysis induced by this subunit. This supernatant was treated with 33% saturated ammonium sulfate to precipitate the residual antibody to C8 $\beta$ , which was then dialyzed against PBS. The F(ab')<sub>2</sub> antiwhole C8 and anti-C8 $\alpha$ - $\gamma$ , and the IgG anti-C8 $\beta$ , prepared in this manner were used in the experiments on inhibition of hemolysis.

**Inhibition of hemolysis.** The specificity of the various antisera used in this study was evaluated by their ability to inhibit hemolysis of EAC1-7 upon addition of purified C8 or the  $\alpha$ - $\gamma$ - and  $\beta$ -subunits in the presence of Cordis C9. Briefly, 20 units of purified C8 or optimal amounts of either C8 $\alpha$ - $\gamma$  or C8 $\beta$  were incubated for 15 min at 37°C with dilutions of the antisera, and inhibition of hemolysis was examined in the hemolytic system described above. The results of the inhibition were referred to controls in which the antisera were omitted. The specificity of the antisera was further confirmed by the ability of purified C8 or of each of the C8 subunits to restore hemolysis to samples in which inhibition of hemolysis had been induced by specific antiserum. In these experiments, the dilution of the antiserum was chosen to give 70-80% inhibition of hemolysis and twice as much C8 or its subunits were used as were present in the initial mixture.

**SDS-PAGE.** Human C8 purified from NHS and immune precipitates from NHS and the two C8-deficient sera (prepared as described above) were subjected to SDS-PAGE. The

discontinuous gel electrophoresis system of Laemmli (16) as modified by Bhakdi et al. (17) with the addition of 6 M urea to the gel was used in these experiments.

**Chromatography of serum.** A small volume (250  $\mu$ l) of NHS, group 1 or group 2 C8-deficient serum, or a 3:1 (group 2/group 1) mixture of the two C8-deficient sera was applied to an 83  $\times$  1.5-cm Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ) column at 4°C. Elution was performed in 0.01 M phosphate buffer (pH 7.4) containing 0.15 M NaCl. Fractions (1.7 ml) were collected and the protein content determined by the absorbance at 280 nm. Hemolytic activity in each of the fractions was assessed using three separate assay systems: (a) EAC1-7 + C9 to detect whole C8; (b) EAC1-7 + R.G. (group 2) serum to detect whole C8 and the C8 $\alpha$ - $\gamma$ -subunit; and (c) EAC1-7 + D.T. (group 1) serum to detect whole C8 and the C8 $\beta$ -subunit. The peak of protein activity corresponding to IgG (determined by double-immunodiffusion analysis of the fractions against goat anti-human IgG) was used as a reference point, to assure accurate comparison of the chromatographic patterns obtained with the different sera.

## RESULTS

**Evaluation of the hemolytic activity of C8-deficient sera.** The present investigation was prompted by our initial observation that lines of hemolysis developed in hemolytic plates between adjacent wells filled with two different types of C8-deficient sera. Confirmation of this finding came from the analysis of seven C8-deficient sera. Four of these sera (group 1) had dysfunctional C8 molecules cross-reacting with C8 in NHS, as shown in the immunodiffusion plate in

Fig. 1. Under the same experimental conditions, the three remaining C8-deficient sera (group 2) did not form a precipitin line with anti-C8 (results not shown). When sera from each of these two groups were added to adjacent wells in a hemolytic plate, a line of hemolysis developed between the wells (Fig. 1). A hemolytic reaction occurred only between sera belonging to different groups; sera from the same group failed to initiate hemolysis.

**Reconstitution of hemolytic activity of the sera by purified C8 subunits.** Previous studies have shown that the C8 hemolytic activity of the sera of group 1 was restored by the addition of C8 $\beta$ , but not by C8 $\alpha$ - $\gamma$  (11, 14). Thus, we directed our attention towards the characterization of the defect in the group 2 sera that apparently lacked a dysfunctional molecule as defined by immunodiffusion. The results of reconstitution experiments (Fig. 2) demonstrated that hemolytic activity could be restored to group 2 sera by the addition of C8 $\alpha$ - $\gamma$ . In contrast, the addition of as much as 15  $\mu$ g of C8 $\beta$  failed to restore hemolytic activity to any of these sera (results not shown). For one of the group 2 sera, the restoration of hemolytic activity by C8 $\alpha$ - $\gamma$  was not as complete as for the remaining two; further analysis of this serum was prevented by its limited supply.

**Evidence for the contribution of C8 $\alpha$ - $\gamma$  and C8 $\beta$  by the C8-deficient sera.** Because sufficient quantities of the other sera were unavailable, this experiment and



FIGURE 1 Antigenic and hemolytic analysis of C8-deficient sera. The Ouchterlony plate on the left contained 5  $\mu$ l of rabbit anti-human C8 in the central well. The peripheral wells contained 40  $\mu$ l of NHS or different group 1 C8-deficient sera (1, 3, 5, 7). The hemolytic plate on the right contained 1% EAC1-7 and 1,000 units of C9. The wells were filled with 10  $\mu$ l of different C8-deficient sera (sera 1, 3, 5, 7—group 1; sera 2, 4, 6—group 2). Detailed analyses of these sera have been published previously: serum 1 (11), 2 (6), 3 and 7 (9), 4 R.G. (5), 5 D.T. (13, 14), 6 (8).

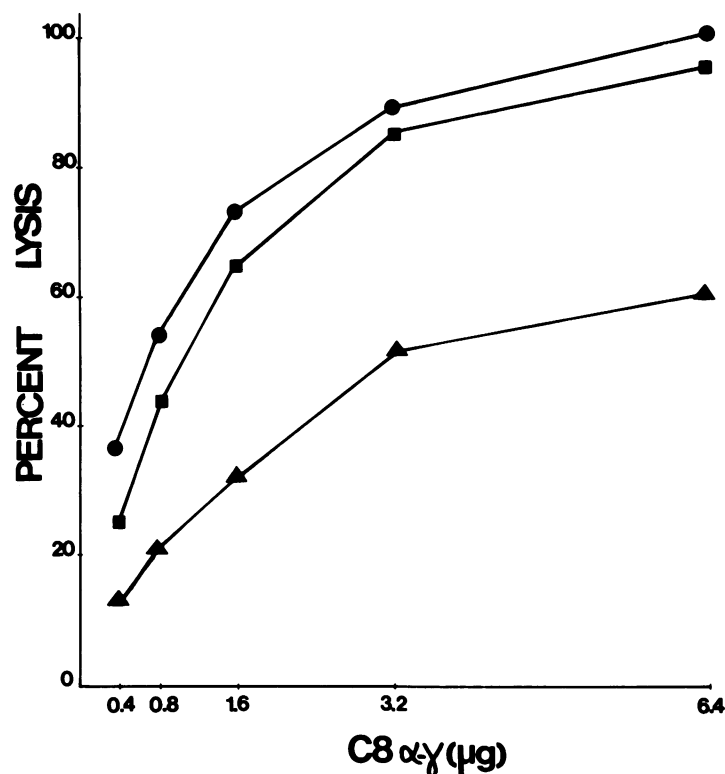


FIGURE 2 Reconstitution of the hemolytic activity of group 2 C8-deficient sera by the purified C8 $\alpha$ - $\gamma$ -subunit. The hemolytic system contained 50  $\mu$ l of EAC1-7 ( $1.5 \times 10^7$  cells), C8-deficient serum (1:20 final dilution), and C9 to which C8 $\alpha$ - $\gamma$  was added in a final volume of 250  $\mu$ l. The C8-deficient sera were obtained from R.G. (●) (5), or kindly provided by Dr. Agnello, Boston (■) (8), and Dr. Jasin, Dallas (▲) (6).

those following were carried out on only two sera: D.T. of group 1 and R.G. of group 2. The experiments in which the hemolytic activity of the deficient sera was reconstituted by the addition of purified subunits (Fig. 2) strongly supported the concept that hemolysis of EAC1-7 by the mixture of the two types of C8-deficient sera was the result of the interaction of C8 $\alpha$ - $\gamma$  and C8 $\beta$  provided by the individual deficient sera. Additional support for this conclusion was obtained by the inhibition of hemolysis induced by specific anti-serum and further reconstitution of lysis by the addition of the missing component. In these experiments, F(ab')<sub>2</sub> anti-whole C8, F(ab')<sub>2</sub> anti-C8 $\alpha$ - $\gamma$ , and IgG anti-C8 $\beta$  were separately added to a mixture of D.T. and R.G. sera. To avoid an excess of antibody to C8 in the system, an amount of antibody was chosen that did not completely inhibit hemolysis of EAC1-7 induced by the mixture of the two deficient sera. D.T. or R.G. serum or a mixture of the two sera was then added to the inhibited samples, and their ability to restore hemolysis of EAC1-7 after 30 min at 37°C was evaluated. The results presented in Fig. 3 show that all three

antibodies were able to inhibit the hemolysis of EAC1-7 by the mixture of D.T. and R.G. sera, as indicated by the low percent lysis in the reaction mixtures treated with each type of antibody. When F(ab')<sub>2</sub> anti-whole C8 was used to inhibit hemolysis in the D.T. plus R.G. mixture, hemolysis could be restored only by the further addition of both D.T. and R.G. sera. When hemolysis in the mixture was inhibited by anti-C8 $\beta$ , hemolysis could be restored by the further addition of R.G. serum but not D.T. serum. When hemolysis in the mixture was inhibited by F(ab')<sub>2</sub> anti-C8 $\alpha$ - $\gamma$ , it could be restored by the further addition of D.T. serum but not R.G. serum. These results indicate that mixing the two types of C8-deficient sera produces hemolytic activity in the mixture. This hemolytic activity can be inhibited by the addition of antibody to the whole C8 molecule or by antibody to either of the C8 subunits. When antibody to whole C8 molecule is used for hemolytic inhibition, hemolysis can be restored only by addition of whole C8 activity. When hemolysis in the mixture is inhibited by antibody to the C8 $\beta$ -subunit, only a source of  $\beta$ -chain (R.G. serum)

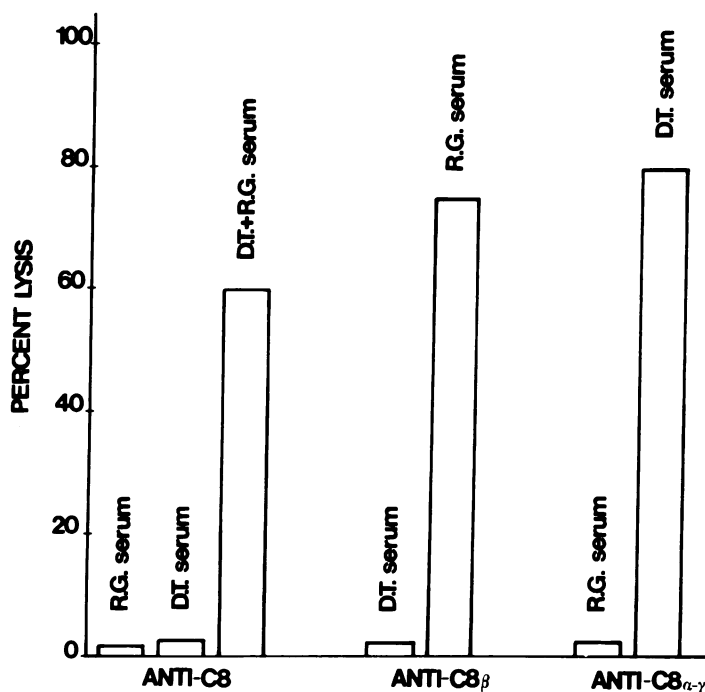


FIGURE 3 Reversal of specific anti-C8 antibody-induced inhibition of hemolysis by further addition of C8-deficient sera. The hemolytic system consisted of a hemolytically active mixture of R.G. and D.T. sera to which specific antibody [F(ab')<sub>2</sub> anti-C8, IgG anti-C8 $\beta$ , or F(ab')<sub>2</sub> anti-C8 $\alpha$ - $\gamma$ ] had been added in sufficient quantity to give 80% inhibition of hemolysis of 50  $\mu$ l of EAC1-7. The columns represent the degree of hemolysis restored to the inhibited samples by the addition of twice the amount of a given C8-deficient serum or a mixture of C8-deficient sera present in the original mixture.

will restore hemolysis. Similarly, when hemolysis in the mixture is inhibited by antibody to the C8 $\alpha$ - $\gamma$ -subunit, only a source of  $\alpha$ - $\gamma$ -subunit (D.T. serum) will restore hemolysis. Thus, R.G. C8-deficient serum lacks the C8 $\alpha$ - $\gamma$ -subunit but contains the C8 $\beta$ -chain, whereas D.T. C8-deficient serum lacks the C8 $\beta$ -chain but contains the C8 $\alpha$ - $\gamma$ -subunit. This conclusion is further supported by the results of SDS-PAGE of immunoprecipitates obtained from NHS and C8-deficient sera after incubation with rabbit anti-human C8 (Fig. 4). Unlike purified human C8 (lane 1) or C8 immunoprecipitated from NHS (lane 2), the C8 precipitated from D.T. (lane 3) or R.G. (lane 4) demonstrated a single band with the mobility of  $\alpha$ - $\gamma$ - or  $\beta$ -subunit, respectively.

**Dose-response curve of hemolysis obtained with C8-deficient sera.** Dose-response curves were established by mixing various dilutions of one of the sera with a constant volume of the other serum and evaluating the hemolytic activity of the mixtures on EAC1-7. As demonstrated in Fig. 5, R.G. serum was the limiting reagent, since higher concentrations of it than of the D.T. serum were required to achieve a comparable degree of hemolysis.

**Binding of the dysfunctional C8 molecules to EAC1-7.** Binding of C8 $\beta$ -subunit to EAC1-7 has been clearly established (3, 14). It was, therefore, of interest to see whether a similar conclusion could be reached

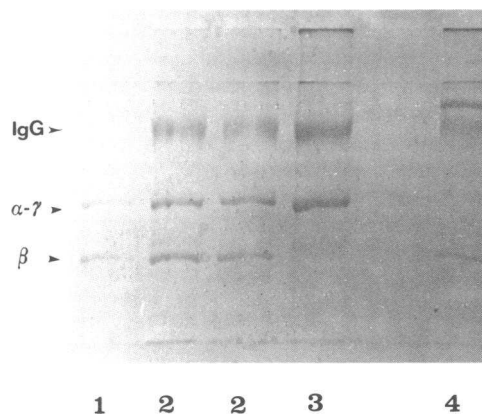


FIGURE 4 SDS-PAGE analysis of purified C8 (lane 1), and of C8 immunoprecipitated from NHS (lane 2) and the sera of D.T. (lane 3) and R.G. (lane 4). The gel was run under nonreducing conditions.

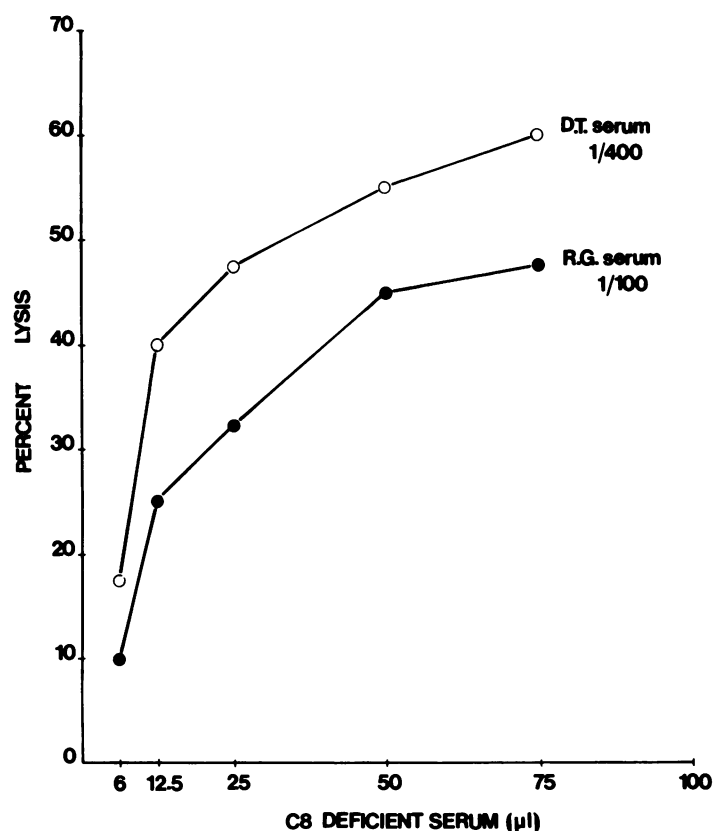


FIGURE 5 Hemolytic activity of mixtures of R.G. and D.T. C8-deficient sera. The hemolytic system contained 50  $\mu$ l EAC1-7, a constant volume (25  $\mu$ l of a 1:10 dilution) of one of the C8-deficient sera, and increasing volumes of the other C8-deficient serum from a starting dilution of 1:100 for R.G. serum and 1:400 for D.T. serum in a final volume of 250  $\mu$ l.

with the C8 subunits in the group 2 C8-deficient sera. For this purpose, EAC1-7 were incubated with one of the C8-deficient sera, thoroughly washed, and then mixed with the second C8-deficient serum. Hemolysis was determined following incubation at 37°C for 30 min. Significant hemolysis occurred only when the cells were first incubated with R.G. serum. No hemolysis was produced when D.T. serum was used as the first reagent even at the highest serum concentrations (Table I).

*Evaluation of the reconstituted C8 molecule by antigenic analysis.* The sera of the C8-deficient subjects, D.T. and R.G., and the mixture of the two sera were examined by immunodiffusion analysis against an anti-C8 antiserum (Fig. 6). As previously reported (13) and as shown in Fig. 1, the precipitin line obtained with D.T. serum (well 2) presents a pattern of partial identity with that of NHS (well 1). A precipitin line cross-reacting with that of NHS was produced also by R.G. serum (well 3) that had previously been reported to lack an antigenically detectable C8 molecule (5).

The two C8 molecules of R.G. and D.T. are complementary and, when mixed (well 4), produce a C8 indistinguishable antigenically from the C8 of NHS (well 1). The assembly of a complete C8 molecule from the dysfunctional C8 molecules required that R.G. and D.T. sera be mixed at a ratio of 3 to 1.

TABLE I

*Evaluation of the Binding of Dysfunctional C8 to EAC1-7*

Cellular intermediate	First reagent	Second reagent	EAC1-7 lysis
			%
EAC1-7	D.T. 1:25	R.G. 10 $\mu$ l	7
EAC1-7	D.T. 1:50	R.G. 10 $\mu$ l	6
EAC1-7	D.T. 1:100	R.G. 10 $\mu$ l	5
EAC1-7	R.G. 1:25	D.T. 10 $\mu$ l	72
EAC1-7	R.G. 1:50	D.T. 10 $\mu$ l	52
EAC1-7	R.G. 1:100	D.T. 10 $\mu$ l	43

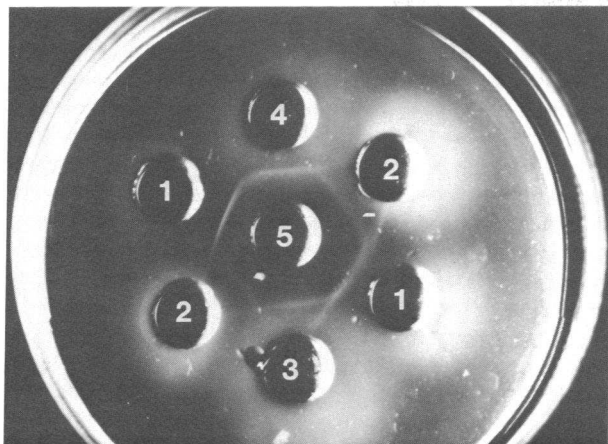


FIGURE 6 Antigenic analysis of NHS and C8-deficient sera by double immunodiffusion with anti-C8 antiserum. The wells and their contents were (1) NHS 35  $\mu$ l; (2) D.T. 60  $\mu$ l; (3) R.G. 200  $\mu$ l; (4) D.T. 60  $\mu$ l plus R.G. 200  $\mu$ l and (5) rabbit anti-human C8 7.5  $\mu$ l. Due to the large volume of R.G. serum used in the assay, the original 200  $\mu$ l were lyophilized and reconstituted with either 60  $\mu$ l of distilled water (well 3) or 60  $\mu$ l of D.T. serum (well 4).

*Evaluation of the reconstituted C8 molecule by column chromatography.* NHS, a mixture of the two types of C8-deficient sera, and the individual C8-deficient sera were chromatographed on a Sephacryl S-200 column. The hemolytic activity in the collected fractions from each chromatographic procedure was assessed in three separate assays that detected (a) whole C8 activity; (b) whole C8 and C8 $\alpha$ - $\gamma$ -subunit activity; and (c) whole C8 and C8 $\beta$ -subunit activity. Hemolytic activity in both NHS and the mixture of the two C8-deficient sera appeared as a peak with an apparent molecular weight in the 150,000 range, consistent with the reported molecular weight (151,000) for C8 (4). This peak of hemolytic activity was the only significant activity detected in the sera and the same peak was detected in all three assay systems. Hemolytic activity in D.T. (group 1, C8 $\beta$ -subunit deficient) serum was detected only in the C8 $\alpha$ - $\gamma$ -subunit assay system. It appeared as a single peak in the 90,000 plus molecular weight range, consistent with the reported molecular weight (99,000) for the C8 $\alpha$ - $\gamma$ -subunit (1). Hemolytic activity in R.G. (group 2, C8 $\alpha$ - $\gamma$ -subunit deficient) serum was detected only in the C8 $\beta$ -subunit assay system. It appeared as a single peak in the <67,000-molecular weight range, consistent with the reported molecular weight (64,000) for the C8 $\beta$ -subunit (4). These results indicate that generation of hemolytic activity in the mixture of the two types of C8-deficient sera occurs as a result of the reconstitution of the whole C8 molecule rather than the sequential action of the  $\beta$ - and  $\alpha$ - $\gamma$ -C8 subunits. This

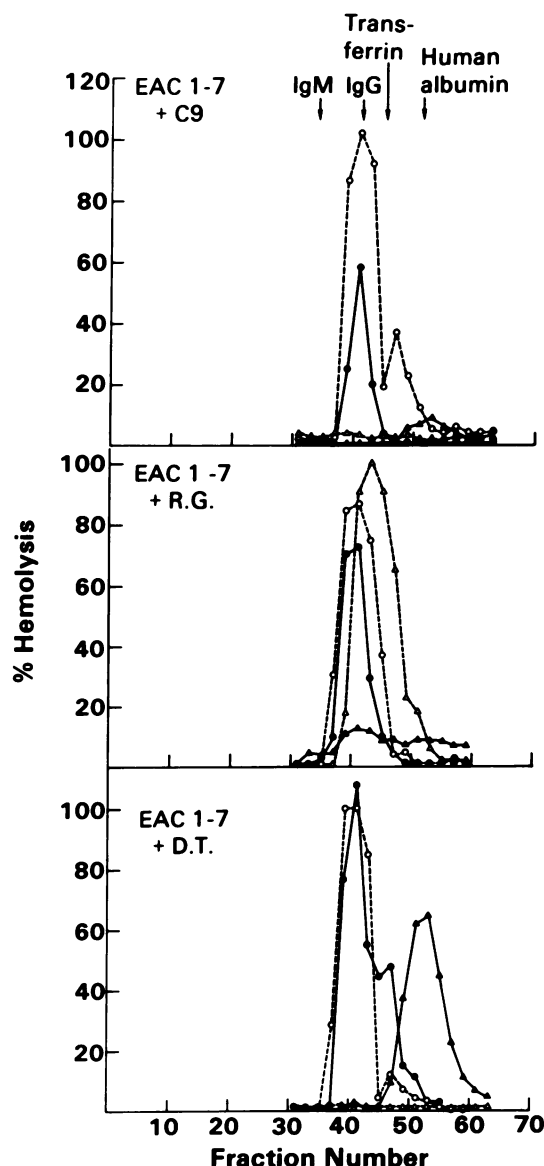


FIGURE 7 C8 hemolytic activity in fractions of chromatographed NHS (O), a mixture of C8-deficient sera (●), and individual C8-deficient sera: D.T. (group 1,  $\beta$ -subunit deficient, [Δ]) and R.G. (group 2,  $\alpha$ - $\gamma$ -subunit deficient, [▲]). Molecular weight markers used were IgM (900,000), IgG (150,000), transferrin (90,000), and human serum albumin (67,000). The assay system EAC1-7 + C9 (top panel) detects only the whole C8 molecule (reported molecular weight 151,000). The assay system EAC1-7 + R.G. serum (middle panel) detects whole C8 and the C8 $\alpha$ - $\gamma$ -subunit (reported molecular weight 99,000), which is absent from R.G. serum. The assay system EAC1-7 + D.T. serum (bottom panel) detects whole C8 and the C8 $\beta$ -subunit (reported molecular weight 64,000), which is absent from D.T. serum.

interpretation is consistent with the reported reconstitution of functional C8 activity from purified C8 subunits (4).



## DISCUSSION

The results of this study show that active C8 can be produced by mixing two different groups of C8-deficient sera, one with a dysfunctional molecule recognized antigenically by an anti-C8 antiserum, and a second group with a different dysfunctional molecule that is not readily detectable by immunodiffusion analysis.

The reconstitution of C8 activity from the two C8-deficient sera was apparent from experiments using hemolytic plates in which lines of hemolysis developed between adjacent wells filled with two different C8-deficient sera. The only missing complement component in this system was C8, since the agarose mixture contained both EAC1-7 and C9. Since no hemolysis was observed in plates containing either C8-deficient serum alone, the development of hemolysis when they were added to adjacent wells on the same plate supports the interpretation that hemolysis results from the interaction of two factors diffusing from adjacent wells. A similar phenomenon has been reported in the reactive hemolysis system where acute phase reactant and NHS provide the source of C5 $\beta$  and C7, respectively (18, 19). The appearance of lines of hemolysis does not contradict the conclusion of previous studies (5, 6, 8, 9, 11, 13), which established the lack of hemolytic activity in these sera. This was confirmed in the present study by the absence of hemolytic rings surrounding the individual wells.

More direct evidence for the formation of active C8 in the mixture of the two C8-deficient sera was obtained from inhibition experiments with an antiserum against the whole human C8 molecule. This antiserum inhibited hemolysis of EAC1-7 induced by the addition of a mixture of the two types of C8-deficient sera. This inhibition could be reversed only by the further addition of both C8-deficient sera, thus confirming the participation of the two sera in the hemolytic process.

The nature of the two factors needed for the reconstitution of active C8 was clarified by three lines of evidence. First, the four sera (group 1) with antigenically detectable dysfunctional molecules acquire hemolytic function after the addition of C8 $\beta$  purified from normal human C8 (14), and the addition of purified C8 $\alpha$ - $\gamma$  to the three C8-deficient sera with no antigenically detectable C8 (group 2) restored hemolytic activity to these sera. Second, antibodies directed against C8 $\alpha$ - $\gamma$  or C8 $\beta$  selectively inhibited hemolysis of EAC1-7 by the sera of groups 1 or 2, respectively. Third, the results of the SDS-PAGE analysis indicate that the two groups of sera contain different subunits of C8; D.T. serum (group 1) contains C8 $\alpha$ - $\gamma$  but lacks the C8 $\beta$ -subunit, while R.G. serum (group 2) contains C8 $\beta$ - but lacks the C8 $\alpha$ - $\gamma$ -subunit. Thus, these exper-

iments clearly support the separation of C8-deficient sera into two groups. In preliminary results using a different procedure, Marcus et al. (20) have recently reached a similar conclusion by showing that there are two patterns of C8 polymorphism that vary according to the source of C8-deficient sera as the revealing reagent.

The experiments concerning reconstitution of hemolysis in which one of the sera was added in constant amounts to the other serum at varying dilutions showed that the limiting reagent was R.G. serum. This serum had to be present in a fourfold greater concentration than D.T. serum in order to achieve optimal hemolysis. Because the C8 subunits are present in an equimolar ratio in the intact molecule, this result probably reflects different levels of C8 $\beta$  and C8 $\alpha$ - $\gamma$  in the two C8-deficient sera with C8 $\beta$  being present at a lower concentration in R.G. serum with respect to C8 $\alpha$ - $\gamma$  in D.T. serum. Experimental support for this assumption requires quantitation of levels of the two subunits in the sera. Unfortunately, we were unable to quantitate these levels. However, indirect support for this interpretation is provided by the immunodiffusion analysis of these sera with an anti-C8 antiserum. A precipitin line was produced by R.G. serum when a considerably larger volume than that required for D.T. serum was used in the Ouchterlony plates. By contrast, lower volumes of R.G. serum did not give visible precipitin lines, explaining the conclusion of a previous study (5) that the C8 molecule was not detectable in this serum.

An alternative explanation might be that in the intact C8 molecule, C8 $\beta$  is less active immunogenically than C8 $\alpha$ - $\gamma$ . If this were the case, antibody generated by immunization with whole C8 might not readily detect the C8 $\beta$ -subunit in the group 2 sera but would detect the C8 $\alpha$ - $\gamma$ -subunit in group 1 sera. This explanation would not account for the finding that R.G. serum was the limiting reagent in the hemolysis reconstitution experiments. However, since this serum contains C8 $\beta$  and since it is the  $\beta$ -subunit that binds to C567 the limiting nature of R.G. serum might be due to the possibility that an actual C8 molecule is not formed upon mixing R.G. and D.T. serum; rather, the two subunits react sequentially to restore hemolytic activity analogous to the experiments in Table I. Under these conditions, R.G. serum would indeed be the limiting reagent. However, the observation that the mixture of R.G. and D.T. sera produce a precipitin line against anti-C8 that exhibits a pattern of complete identity with the precipitin line in NHS and the results of the chromatography experiments indicate that the C8 molecule is, in fact, immunogenically and functionally reconstituted.

In conclusion, we have documented the existence

of two groups of C8-deficient subjects carrying in their sera one of the two C8 subunits, C8 $\alpha$ - $\gamma$  or C8 $\beta$ . We have also demonstrated that the mixture of sera from the two groups allows the reconstitution of the function and the antigenic integrity of the C8 molecule.

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