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Identification of the Heterozygotes for Deficiency of the β-Subunit of the Eighth Component of Complement by Reduced Levels of C8β and Increased Amounts of Free C8α-γ[1]

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ABSTRACT. Sera from obligate heterozygotes for deficiency of the C8β subunit of the eighth component of human complement (C8) were analyzed for the molecular composition of C8. The C8α-γ and C8β subunits were separated by SDS-PAGE, visualized by immunoblotting, and the resulting bands were quantitated by laser densitometry. The laser densitometric absorption data were set to 100 arbitrary units (AU) for both subunits of pooled normal human sera. The AU values of individual normal sera ranged from 45 to 150 AU for C8α-γ (median 99 AU) and from 45 to 140 AU for C8β (median 101), whereas the C8α-γ/C8β-ratio varied from 0.7 to 1.4. Sera from C8β-deficient heterozygotes differed, as expected, from the normal sera for the markedly reduced levels of C8β (20 to 90 AU, median 55 AU) and for the higher C8α-γ/C8β-ratio (1.3 to 3.5). High voltage agarose gel electrophoresis was used to separate free and C8β-bound C8α-γ. The migration of free and C8β-bound C8α-γ subunit was checked by hemolytic overlay gels and by second dimension SDS-PAGE and immunoblotting. Immunochemical evaluation of C8α-γ using this system revealed about 5–14% free C8α-γ in sera with normal C8 and higher levels, from 33–71%, in the C8βD heterozygous sera. Functional analysis confirmed the substantial increase of free C8α-γ in the heterozygous group. We conclude that the C8 in C8βD heterozygous sera is characterized by increased amounts of free C8α-γ due to reduced concentrations of the C8β subunit. This finding may help to identify individuals heterozygous for C8β deficiency. (*Pediatr Res* 27: 234–238, 1990)

Abbreviations

C8, eighth component of human complement
C8βD, C8β deficient
C8 α-γD, C8α-γ deficient
GVBS, glucose veronal buffered saline
HVAE, high voltage agarose gel electrophoresis
NHS, normal human serum
PNHS, pooled normal human serum
AU, arbitrary unit

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The congenital deficiency of the β-subunit of the eighth component of the complement system (C8) has been reported in more than 40 individuals (1). The diagnosis of the C8β defect can easily be made for the homozygotes whose sera lack C8 lytic activity. These sera contain an immunochemically detectable C8α-γ subunit cross-reacting with the C8 of NHS with pattern of partial Ag identity (2, 3). The C8α-γ present in C8β-deficient sera, however, is not dysfunctional because it is still capable of reacting with the β-subunit to form a hemolytically active C8 molecule (4, 5). Unlike the homozygotes, the individuals heterozygous for C8β deficiency are somewhat more difficult to recognize due to the broad range of antigenic and hemolytic values of C8 in control populations. Search for null genes in family studies may be of some help when positive, but this is not always the case (6).

A more practical approach to screen for heterozygotes for C8β deficiency is provided by the findings of Brandslund *et al.* (7). The authors showed two anti-C8 reacting components with α- and β-mobility in normal human plasma, whereas the β-component was absent in the plasma obtained from one C8-deficient patient later recognized to have a C8β defect (F. Tedesco and I. Brandslund, unpublished observation), and was reduced to half normal levels in the plasma of both parents and two siblings of the propositus. This study, however, did not clarify the molecular nature of the two components with respect to the subunit composition of C8 and it was not extended to include other families with a similar defect.

The purpose of our investigation is to show that the heterozygotes for C8β-deficiency can easily be recognized by measuring the α-γ- and the β-subunit of C8. In addition, evidence will be provided, indicating that these subjects present increased levels of free C8α-γ in their sera as compared to the control population.

MATERIALS AND METHODS

Sera. Blood samples were collected from 32 normal individuals, five homozygous C8βD patients, and the 10 parents of these patients, allowed to clot at room temperature for 30 min, and stored in small aliquots at −80°C. Control sera were obtained from healthy laboratory personnel and blood donors, and were

used either individually (NHS) or as a pool of equal volumes of all sera (PNHS). Details of the C8βD patients have been given in previous reports (3, 5, 8, 9). A serum sample of a C8α-γD patient was a generous gift from Dr. B. Petersen (Lilly Laboratory for Clinical Research, Indianapolis, IN). Sera from abroad were shipped on dry ice. Complement activation in the sera was checked by determining the C3dg concentration as described previously (10).

Antisera. Four polyclonal antisera against the whole C8 molecule were obtained from the following sources: A, goat anti-human C8 (Cappel, Cochranville, PA); B, goat anti-human C8 (Atlantic antibodies, Scarborough, ME); C, rabbit anti-human C8 (Calbiochem, La Jolla, CA); and D, rabbit anti-human C8 (our own preparation) (4). The antibodies A and C were diluted 1 in 2000, the antibodies B and D in 1 in 1000. Sources of alkaline phosphatase-labeled antibodies were goat anti-rabbit IgG (Jackson-Dianova, Hamburg, FRG) diluted 1 in 4000, and rabbit anti-goat IgG (Sigma, Munich, FRG) diluted 1 in 1200.

SDS-PAGE and immunoblot analysis of C8. The previously described procedure for the analysis of the C8 subunits (5) was followed with minor modifications. These include the electroblotting at 1.2 mA/cm^2 gel surface for 70 min using a semi-dry blotting apparatus (11) and a continuous buffer system (12).

Laser densitometric evaluation of C8α-γ and C8β subunits. The immunostained C8α-γ and C8β bands were quantitated by laser densitometry using a neon-helium laser at 633 nm wavelength (UltroScan 2202, Pharmacia, Bromma, Sweden). Inasmuch as the C8α-γ subunit is polymorphic and gives two or three bands in different sera (5), the absorption values of the individual bands were added together to obtain the total amount of the C8α-γ subunit. The C8α-γ and C8β values were expressed as AU, which were calculated considering equal to 100 AU for both the C8α-γ and the C8β subunit of PNHS diluted 1 in 60. Consequently, all the samples to be tested were diluted 1 in 60, and their absorption values were referred to a standard curve constructed with dilutions of PNHS ranging between 1 in 15 to 1 in 480 (in 150 mM sodium chloride) assayed in parallel. Although the shape of the standard curve was sigmoidal for both C8α-γ and C8β, the absorption values were linear over a dilution range of PNHS between 1 in 30 and 1 in 240. The calculated C8α-γ/C8β-ratio was stable in the range from 25 AU to 200 AU for PNHS. The C8α-γ/C8β ratio was calculated as follows:

$$\text{C8}\alpha\text{-}\gamma/\text{C8}\beta \text{ ratio} = \frac{\text{C8}\alpha\text{-}\gamma(\text{AU})}{\text{C8}\beta\ (\text{AU})}$$

HVAE. A 0.5-mm agarose gel was prepared by mixing equal volumes of agarose type A (Calbiochem) with an electroendosmosis of −0.13 M_r in distilled water and Tris-glycine buffer, pH 9.6, to a final concentration of 1% agarose, 180 mM Tris, 90 mM glycine, and a conductivity of 1000 μs/cm at room temperature. The mixture was prewarmed to 60°C, poured between two glass plates (12.5 × 16 cm), one of which had been covered with a Mylar cellophane sheet (LKB, Bromma, Sweden) and left to solidify at 4°C for about 2 h. Electrophoresis was performed at 10°C using the LKB apparatus (Multiphore 2117) with a 30-min prerun at 15 V/cm. The 1- to 2-μL samples were then applied to 0.6 × 7 mm slots in the agarose gel and, after a 30-min run at 6 V/cm, the voltage was adjusted to 40 V/cm. The electrophoresis was stopped when Hb, used as a marker, had migrated 8 cm from the application side, which was reached after a 60 to 70 min run. The agarose gel was then processed either for a second dimension SDS-PAGE and subsequent blotting onto nitrocellulose or for the hemolytic overlay to reveal the C8 hemolytic bands.

Second dimension SDS-PAGE. Conditions for the second dimension SDS-PAGE were similar to those reported above. Agarose gel strips from HVAE cut along the direction of the electrophoretic run were rinsed in the sample buffer containing 1.5% SDS and 62.5 mM Tris and deposited on top of the polyacrylamide gel. PNHS (0.5 μL) and a marker solution (Bio-Rad, Richmond, CA) were run in parallel. The C8 bands were electroblotted onto nitrocellulose and subsequently revealed using the anti-C8 antiserum from N. L. Cappel Laboratories, Cochranville, PA. Quantitation was carried out by measuring the area of the stained spots. A linear relationship between size of the spots and serum dilution was found from 3 to 70% for free C8α-γ.

Detection of functionally active C8α-γ and C8β by hemolytic overlay gel. The procedure for the preparation of EAC1-7 using a C8βD serum has been previously reported (3). It is now well established that the C8βD sera contain the C8α-γ subunit that is unable to bind to EAC1-7 (4). A 10% EAC1-7 suspension in GVBS was incubated with 20% (vol/vol) C8α-γD serum or 20% (vol/vol) C8βD serum (for 10 min at 37°C) to prepare erythrocyte intermediates suitable for assaying functional C8β and C8α-γ, respectively. Equal amounts of the erythrocyte intermediates in twice concentrated GVBS and agarose (Indubiose A 37, Réactifs IBF, Villeneuve, France) suspended in distilled water were mixed at 50°C to obtain a final suspension containing 1% erythrocytes and 1% agarose in GVBS. A volume of this mixture sufficient to prepare a 1.3-mm thick overlay was poured onto a prewarmed agarose gel, which had been quickly rinsed with GVBS to remove the electrophoretic buffer after completion of HVAE. The gels were kept in a humid chamber for 3–4 h at 37°C until the hemolysis was visible. Further lysis was prevented by soaking the gels in 2% glutaraldehyde in 150 mM sodium chloride at 4°C for 30 min.

Statistical analysis. The coefficient of variance and the Pearson's correlation coefficient were calculated using standard programs (Hewlett-Packard Co., Palo Alto, CA).

RESULTS

Standardization of SDS-PAGE and blotting procedure. Initial experiments were performed to find the optimal conditions for a correct quantitation of C8α-γ and C8β subunits in human sera. Various attempts were made to select the transfer time that allowed quantitative transfer of C8 subunits from the polyacrylamide gel to the nitrocellulose sheet. The optimal transfer time was found to be 70 min. To prevent differences in the reaction of the various polyclonal antisera for each of the two subunits from leading to erroneous results, four anti-C8 antisera were examined and the Cappel antiserum was chosen to give the best estimate of the C8α-γ and C8β values. The interassay coefficient variance was 13.6%. Representative patterns and laser densitometric profiles of the C8 subunits in PNHS and in two sera obtained from a homozygote and a heterozygote for C8β deficiency are presented in Figure 1. As expected, C8β was undetectable in the homozygote and substantially reduced in the heterozygote when compared to the C8β level in PNHS. Analysis of four additional sera from patients homozygous for C8β deficiency confirmed the defect of C8β and the presence of C8α-γ, though the latter showed some quantitative variations among patients.

Quantitation of C8α-γ and C8β in C8βD heterozygotes. The SDS-PAGE and blotting procedure was used to examine the sera of the 10 parents of the C8βD patients and, for comparison, those of 32 control sera for their C8α-γ and C8β levels with the intent to recognize the heterozygous state. The results shown in Figure 2 indicate that the two groups had similar values of C8α-γ ranging between 45 and 150 AU, and differed markedly for the levels of C8β. The C8β values in the control group ranged from 45 and 140 AU, whereas eight of the heterozygotes, including the five mothers, had C8β levels less than 40 AU and the remaining two had C8β values in the lowest range of control values. The heterozygotes also differed from the controls in the C8α-γ/C8β ratio, which did not exceed 1.38 in the control sera but rose to 1.7 and more in the majority of the heterozygotes. In the normal population, a strong correlation was found between the C8α-γ and the C8β-values, which was not detectable in the

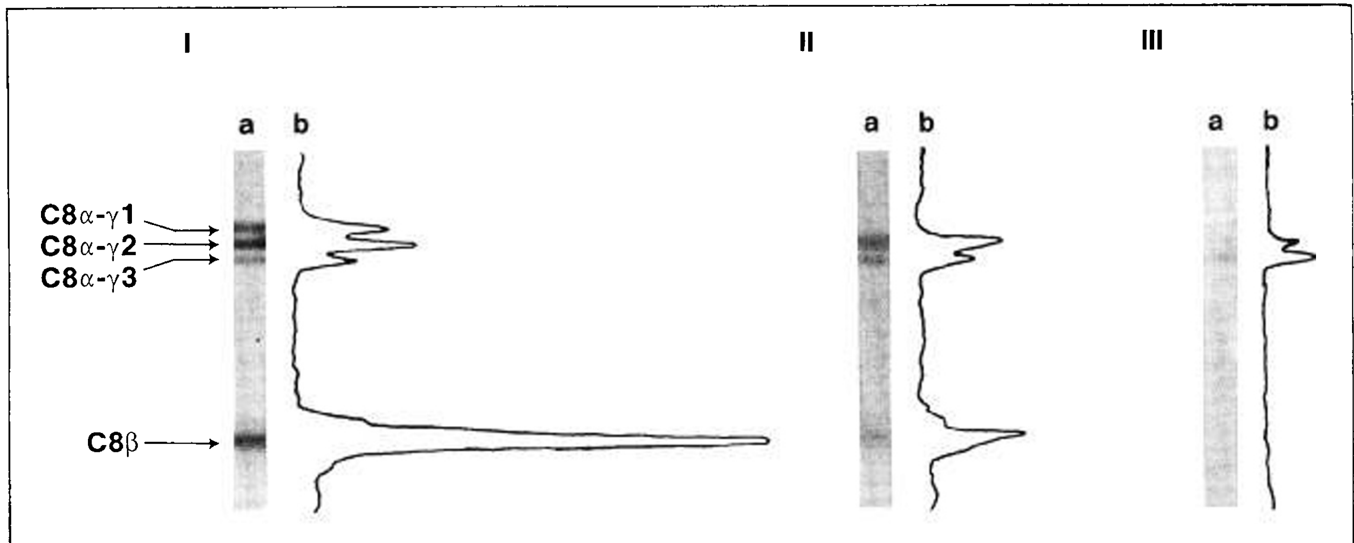

Fig. 1. Representative C8-immunoblots (*a*) and laser densitometric profiles (*b*) of PNHS (*I*), heterozygous C8βD (*II*), and homozygous C8βD serum (*III*) are shown. The position of the C8α-γ subunit (consisting of C8α-γ1, C8α-γ2, and C8α-γ3) and of the C8β subunit are given on the *left*. The C8β subunit is present in the C8βD heterozygous serum (*II*) in considerable lower amounts than in PNHS (*I*) and is completely absent in C8βD homozygous serum (*III*).

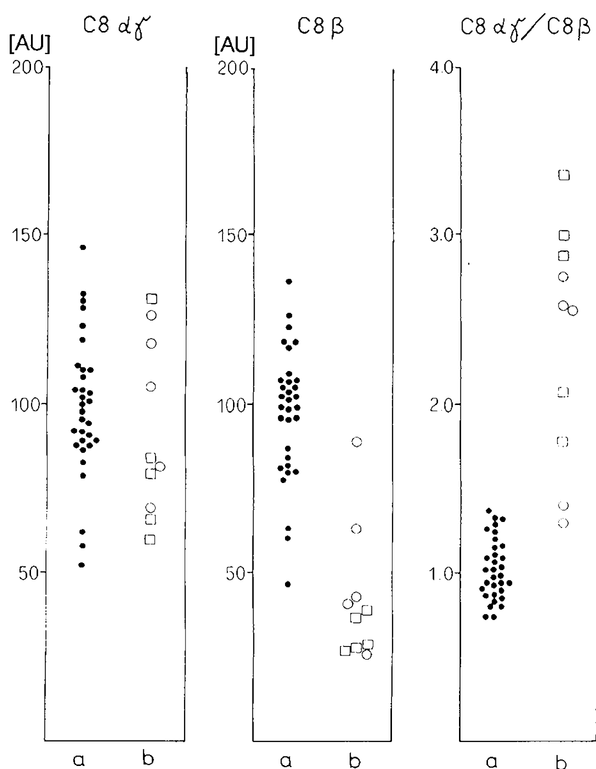

Fig. 2. Laser densitometric quantitation of C8α-γ (*left*), C8β (*center*), and calculated C8α-γ/C8β-ratio (*right*) in 32 normal individuals (*a*) and 10 parents of C8β deficient individuals (*b*); (○, fathers; □, mothers). All values are expressed as arbitrary units (*AU*).

heterozygous group (for normal individuals: $n = 32$, $r = 0.64$, $p < 0.001$; for C8βD heterozygotes: $n = 10$, $r = 0.54$, $p > 0.05$; Pearson's correlation). Inasmuch as separate experiments had shown that immunostaining of C8β tends to be weaker after *in vitro* complement activation by inulin, all sera were tested for the levels of C3dg, which did not differ between controls and heterozygotes and was not higher than 3% (data not shown).

Free C8α-γ is increased in heterozygotes. The relatively high ratio of C8α-γ to C8β in the heterozygous group may be explained by the presence of increased amounts of free C8α-γ in the sera of these individuals. To test this hypothesis, HVAE of heterozygous and control sera was carried out and functional C8α-γ was detected by the hemolytic overlay system using EAC1-C8β plus C9 as described in Materials and Methods. Figure 3 demonstrates that two distinct areas of lysis were detected in NHS showing relative electrophoretic mobilities of 20–50 and 60–90% with respect to that of Hb. The cathodal lytic area was the only one observed when both NHS and C8α-γD serum were tested with the erythrocyte intermediate prepared with a C8βD serum (data not shown). Conversely, the C8βD serum exhibited only the anodal lytic area (Fig. 3, *lane a*). From these results we concluded that the cathodal lytic area reveals the whole C8, whereas the anodal lytic area only shows free C8α-γ. The control and the heterozygous sera showed different lytic patterns when examined with EAC1-8β and C9. Thus, the cathodal area was more intense in the controls, whereas the anodal area was more prominent in the heterozygous group (Fig. 3).

To confirm that the two lytic areas of NHS seen after HVAE correspond to complete C8 and free C8α-γ, two-dimensional electrophoresis was performed using HVAE followed by SDS-

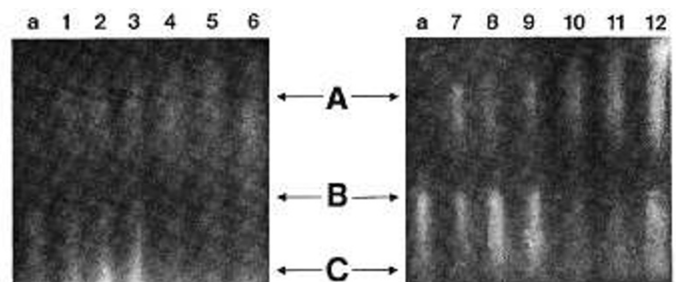

Fig. 3. Functional analysis of C8α-γ separated by HVAE revealed by hemolytic overlay gels (see Material and Methods). Cathode is on the top, the position of the Hb at the end of the electrophoretic run is indicated by C. The figure shows the resulting lytic patterns (zones of hemolysis are white) for a C8βD homozygous serum (*a*), C8βD heterozygous sera (1, 2, 3, 7, 8, 9) and normal control sera (4, 5, 6, 10, 11, 12). Zone A represents the lytic area of C8α-γ bound to C8β (whole C8), whereas zone B corresponds to that of free C8α-γ. The C8βD homozygous serum lacks the slowly migrating C8α-γ (zone A), which is bound to the C8β subunit (see Fig. 4). C8βD heterozygotes and normal individuals show two zones of lysis (*A*) and (*B*). Note, that the lytic zone B is stronger in C8βD heterozygous than in the normal control sera.

PAGE under nonreducing conditions and subsequent blotting. As shown in Figure 4, on SDS-PAGE the cathodal area of NHS resolved into two bands with molecular mass of 87 and 64 kD, which represent C8α-γ and C8β, respectively, whereas the anodal area gave only the 87-kD band. A pattern similar to that of NHS was observed with the serum of a heterozygous individual, whereas the serum from a C8βD homozygous patient only showed the 87-kD band corresponding to the anodal area on HVAE.

To quantitate the relative amounts of free and C8β-bound C8α-γ, sera from 10 heterozygous and nine control subjects were examined by two-dimensional electrophoresis and subsequent immunoblotting. The results presented in Table 1 indicate that free C8α-γ represents about 5–14% of the total C8α-γ and also that the amount of free C8α-γ increases in the heterozygotes to reach values equal or higher than those of the C8β-bound form.

DISCUSSION

SDS-PAGE and immunoblotting procedure has been shown to be useful for measuring C8α-γ and C8β in human sera (5). We now show that it allows recognition of most of the heterozygotes for C8β deficiency. In the Norwegian population, 5–10% were considered to be heterozygous for C8β deficiency (13).

The wide range of C8α-γ and C8β values observed in the control group are in accordance with results obtained in normal human sera with the immunochemical and hemolytic evaluation of the whole C8 molecule (3). Variability in the transfer of the two subunits from the polyacrylamide gel to the nitrocellulose sheet is unlikely to account for these results because the coefficient of variance was lower than 15% on repeated examination of the same sera. Although normal values of C8α-γ and C8β in sera vary over a wide range, our results show a highly significant correlation between the amounts of C8α-γ and that of C8β. The possible existence of a common regulatory control for the synthesis of the two subunits is suggested, although not formally proven, by studies of C8 polymorphism showing close linkage of the two different loci encoding the α-γ- and the β-subunit (14). In addition, studies by Ng and Sodetz (15), on the biosynthesis of C8 by rat hepatocytes, have clearly shown that C8α-γ and C8β, although separately produced, have a preferential tendency to assemble intracellularly as whole C8 due to the high affinity of C8α-γ and C8β. The suggestion has therefore been made that the synthesis of the two subunits is regulated to allow maximal formation of the assembled molecule (15, 17). Whatever the mechanism responsible for the regulatory control of C8α-γ and C8β production, this is certainly not working in the heterozygotes and in the homozygotes for C8β deficiency. In these individuals, the serum concentration of C8α-γ is independent from that of C8β. The low levels of C8β in the heterozygous group confirm and extend previous observations by Brandslund *et al.* (7), who analyzed the sera of family members of a C8βD patient by cross-immunoelectrophoresis. The advantage of our experimental approach is that it allows the definition of the relative amounts of the two subunits of C8 without the requirement of subunit-specific antibodies.

The results of our studies also indicate that the reduced secretion of C8β in the heterozygotes is accompanied by a corresponding increase in free C8α-γ. Evidence for the presence of free C8α-γ in human sera has been obtained using subunit-specific MAb (16) and electrophoretic analysis or exchange chromatography of the sera (17). A similar conclusion was drawn by Ng and Sodetz (15) in their study on C8 biosynthesis showing extracellular secretion of free C8α-γ by rat hepatocytes. Our results are in agreement with those of Doglio *et al.* (18) who demonstrated small amounts of free C8α-γ in sera with normal C8 and substantial levels of this subunit in the sera from C8βD heterozygotes. Free C8α-γ retains its fully hemolytic activity and this is even increased in the C8βD heterozygotes compared to the activity of the C8β-bound C8α-γ. The *in vivo* role of free

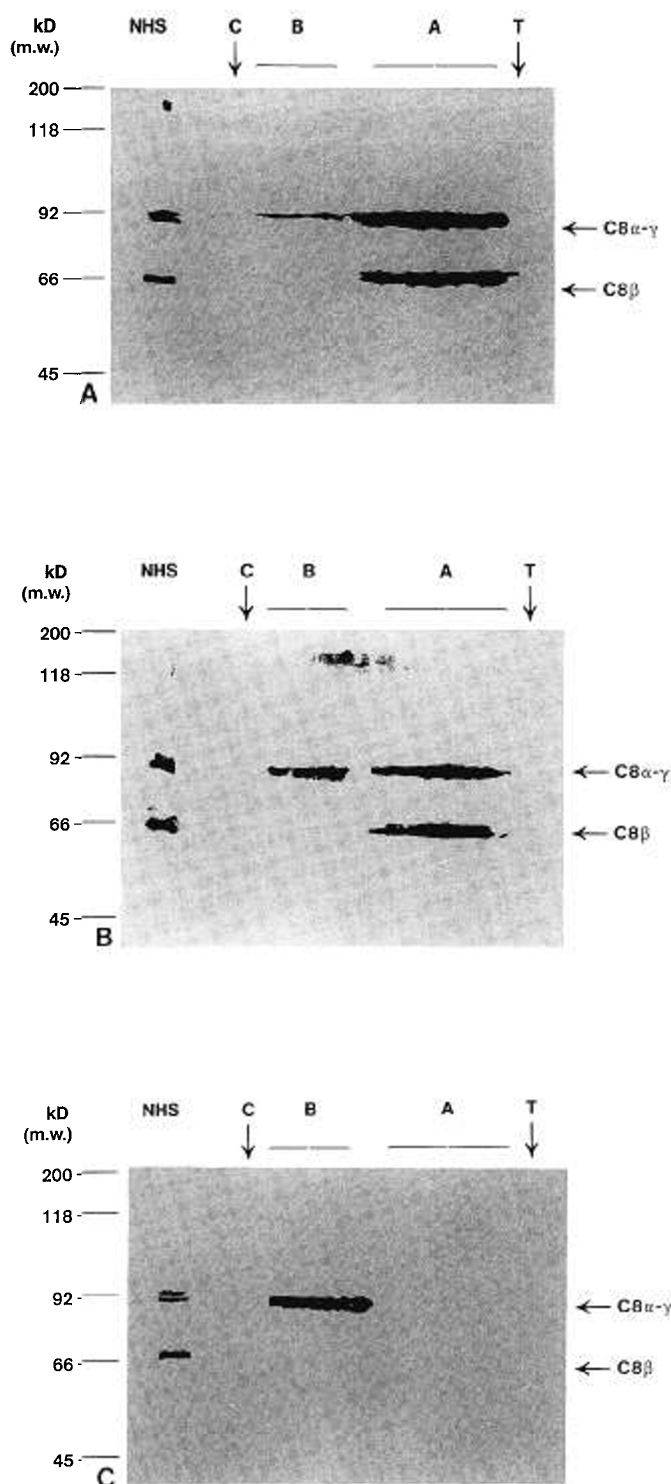

Fig. 4. Patterns of two-dimensional electrophoresis (first dimension HVAE, second dimension SDS-PAGE) of 1 μL NHS (*A*), 1 μL C8βD heterozygous sera (*B*), and 1 μL C8β-deficient serum (*C*). The mol wt markers (*m.w.*) are indicated on the *left*, the positions of the C8α-γ and C8β subunit on the *right*. *NHS*, normal human serum, analyzed only by SDS-PAGE as control for the C8α-γ and C8β position; *T*, position, where the sample was applied for one-dimensional electrophoresis; *C*, position, to which Hb was allowed to migrate in the first dimension; *A*, region of slowly migrating C8, which is composed of C8α-γ and C8β; *B*, region of fast migrating C8, which shows a C8α-γ band only.

Table 1. *Free C8α-γ versus C8β-bound C8α-γ in sera from normal and C8β D-heterozygous Individuals**

Individuals	Free C8α-γ (mm^2)	C8β-bound C8α-γ (mm^2)	Free C8α-γ / (free C8α-γ + C8β-bound C8α-γ)
Normals			
1	14	136	0.05
2	7	140	0.09
3	18	151	0.11
4	13	141	0.09
5	8	126	0.06
6	15	94	0.14
7	8	157	0.05
8	10	138	0.07
9	10	131	0.07
Range	7–18	94–157	0.05–0.14
Median	10	138	0.07
C8β heterozygotes			
1	49	67	0.42
2	70	29	0.71
3	51	57	0.47
4	43	87	0.33
5	65	31	0.63
6	67	53	0.56
7	74	63	0.54
8	84	40	0.68
9	44	85	0.35
10	55	57	0.49
Range	49–84	29–87	0.33–0.71
Median	60	57	0.51

* C8 was separated by two-dimensional electrophoresis (see Fig. 4). The immunostained C8α-γ spots were quantitated by measuring the area (mm^2). Increased amounts of free C8α-γ were found in the sera from obligate C8β-heterozygotes, whereas the C8β-bound C8α-γ is lowered.

C8α-γ remains to be elucidated. The recent finding by Luzio and Stanley (19), of a sequence homology of C8γ with α1-microglobulin, raises the possibilities of other functions of C8α-γ in addition to its well known role in the assembly of the terminal complement complex.

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