Two distinct abnormalities in patients with C8 alpha-gamma deficiency. Low level of C8 beta chain and presence of dysfunctional C8 alpha-gamma subunit.

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Research Article

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Two Distinct Abnormalities in Patients with C8α-γ Deficiency

Low Level of C8β Chain and Presence of Dysfunctional C8α-γ Subunit

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Abstract

The sera from three C8α-γ deficient patients previously reported to have a selective C8α-γ defect were analyzed by SDS-PAGE and Western blot using two polyclonal antisera to C8α-γ and a monoclonal antibody to C8α. All three sera exhibited C8α-γ bands that dissociated into α and γ chains under reducing conditions. Quantitation of the α-γ subunit in these sera by a sensitive ELISA revealed an amount ~ 1% of that found in normal human serum. A similar assay performed with a specific antiserum to C8β showed unexpectedly low levels of C8β in these sera, which were confirmed by hemolytic titration of C8β. The remarkable differences between C8α-γ and C8β in the C8α-γ deficient sera was that in spite of their comparable immunochemical levels, C8β still exhibited functional activity whereas C8α-γ was totally inactive. That the residual C8α-γ was inactive was also proved by its inability to show lytic bands in an overlay system after SDS-PAGE and subsequent removal of SDS. The implications of these findings for a novel concept of C8 deficiency are discussed. (*J. Clin. Invest.* 1990. 86:884–888.) Key words: complement • C8 • inherited defect • C8 subunits • combined deficiency

Introduction

The selective defect of the eighth complement component (C8)[1] is relatively common among the inherited deficiencies of the components and regulators of the complement system (1). Patients with this defect show an increased susceptibility to meningococcal meningitis, indicating that C8 plays an important role as a component of the membrane attack complex and in the protection against neisserial infections (1, 2).

A distinctive feature of C8 deficiency is the presence of a dysfunctional C8 molecule in all the sera of C8-deficient (C8D) subjects that have been tested (2). This is rarely observed in other complement deficiencies with the exception of a small group (10–15%) of patients lacking functional C1-inhibitor (3). On the basis of reconstitution experiments and immunochemical analysis, two forms of dysfunctional C8, and consequently two groups of patients have been identified (4, 5). One form is associated with a dysfunction in the α-γ subunit and the other with a dysfunction in β. The absence of C8 activity in both groups is consistent with the fact that α-γ and β have specific functional roles and both are essential for normal C8 hemolytic activity (6). For reasons that are presently not clear, the inherited defect of C8β is largely predominant over that of C8α-γ and is essentially restricted to Caucasians, whereas the C8α-γ deficiency has been found exclusively in Blacks and North Africans (1, 2).

The possibility that C8 deficiencies result from the complete absence of a particular subunit is unlikely in view of recent findings by Tschopp and co-workers (7). Two C8β-deficient patients were found to have detectable but dysfunctional C8β in their serum along with normal α-γ. Partial characterization indicated that the C8β was structurally abnormal. Thus, at least in some patients, dysfunctional C8β is synthesized but in an altered form. Presumably this altered form is incapable of association with α-γ to produce functional C8.

In this study, we examined in more detail the molecular abnormality of the dysfunctional C8 in three C8α-γD individuals and provide evidence for a further complexity of this inherited defect. Data presented indicate that C8α-γ deficiency is a combined defect of both α-γ and β, and that a small amount of dysfunctional C8α-γ can be detected in the sera of C8α-γ deficient patients.

Methods

Sera. Three unrelated C8α-γD patients and two siblings with C8βD, reported in previous publications (8–11), provided serum samples for this investigation. The type of C8 deficiency was recognized by hemolytic reconstitution with the missing subunits and by identification of the subunit present in the various sera by SDS-PAGE and immunochemical procedures (4). A pool of normal human sera (NHS) was prepared by mixing equal volumes of sera obtained from 20 blood donors.

Complement reagents. Human C8 was purified following the procedure described in detail by Steckel et al. (12). The two subunits, C8α-γ and C8β, were dissociated from C8 in the presence of buffers of high ionic strength and further purified by gel filtration through Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden), as reported by Rao and Sodetz (13).

Erythrocyte intermediates and hemolytic assays. The preparation of EAC1-3 and EAC1-7 from IgM sensitized sheep red cells using yeast-treated human serum and a C8βD serum has been previously described (10). The hemolytic assays for C8α-γ and C8β were per-

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1. *Abbreviations used in this paper:* C8, eighth component of complement; C8D, C8α-γD, and C8βD, C8-, C8α-γ-, and C8β deficient; EAC1-3, EAC1-7, and EAC1-8, sheep erythrocytes coated with antibody and complement components up to C3b, C7, and C8; NHS, normal human sera.

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formed by incubating a mixture of 200 μl of various sera dilutions and 50 μl of EAC1-7 (1.5×10^7), suspended in either C8α-γD or C8βD sera, to a final dilution of 1/50 for 30 min at 37°C. Preliminary experiments proved that the concentrations of the C8D sera used for these assays were not limiting for an optimal titration of the two C8 subunits.

Antisera. A goat antiserum against whole human C8 was purchased from Atlantic Antibodies (Scarborough, ME). Antibodies to C8α-γ were prepared in two different ways. A goat antiserum prepared against whole human C8 was passed through an agarose-(α-γ) affinity column to isolate (α-γ)-specific Ig (14). A second antiserum with a higher avidity for α-γ was obtained by immunizing rabbits with purified α-γ and passing the antiserum through an agarose-(β) column to remove traces of anti-β. Specific antibodies against β were prepared similarly in rabbits using purified β as the immunogen. Specificity was tested by Ouchterlony and SDS-PAGE immunoblotting.

A mouse monoclonal antibody was obtained following an established procedure (15) and found to recognize the α chain of C8. Ascitic fluid was produced in pristane-primed Balb/c mice and used for the experiments.

Biotin labeling of antibodies. The IgG fraction of the rabbit antisera to C8α-γ and C8β was obtained by affinity cromatography through protein A-agarose (Zymed Laboratories, San Francisco, CA) using 0.2 M phosphate/0.1 M citrate pH 3.5 as eluting buffer. The IgG were dialyzed against 0.2 M Tris-HCl pH 8.0 and mixed with D-biotin-*N*-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, MO) (2 mg/ml in DMSO) at a ratio of 1:4 (wt/wt) for 15 min at room temperature. The excess biotin was removed by dialysis against 10 mM PBS pH 7.4 containing 0.01% NaN3.

SDS-PAGE and immunoblotting. Normal and C8D sera were subjected to SDS-PAGE on a 10% gel under nonreducing conditions according to Laemmli (16), followed by electrophoretic transfer onto nitrocellulose (Schleicher & Schuell Inc., Dassel, FRG). After soaking the nitrocellulose sheet in 50 mM Tris-HCl pH 7.6 containing 0.5 M NaCl Tris buffered saline, 2% bovine serum albumin, and 0.5% Tween 20 for 1 h at 37°C to block the free binding sites, the C8 bands were revealed by successive incubations with the primary antibodies to C8 or C8 subunits overnight at 4°C, followed by the biotin-labeled secondary antibodies (Zymed Laboratories) for 2 h at 37°C and alkaline phosphatase conjugated to streptavidine (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at 37°C. The enzymatic reaction was developed using nitroblue tetrazolium (0.30 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.15 mg/ml) (both purchased from Sigma Chemical Co.), and diluted in 0.1 M Tris-HCl pH 9.5 containing 0.15 M NaCl.

Hemolytic overlay. The functional activity of the C8α-γ subunit electrophoresed by SDS-PAGE was revealed following the procedure developed by Orren et al. (17). Briefly, after the electrophoretic run the gels were soaked in an aqueous solution of 0.25% Triton X-100 for 1 h at 4°C to remove SDS and then in H_2O for 1 h at 4°C, followed by a 10-min wash with glucose veronal-buffered saline (18). The gel was covered with a mixture of 1% agarose, 0.6% EAC1-7, and 1/50 C8α-γD serum to obtain a 1-mm thick gel. The overlay was incubated at 37°C in a humid box until the hemolytic bands developed. Further hemolysis was prevented by adding 2% glutaraldehyde in saline.

Quantitation of the C8 subunits by ELISA. The wells of microtiter plates (Dynatech, PBI, Milan, Italy) were coated with 200 μl of the IgG fraction of either anti-C8α-γ (1.6 μg/well) or anti-C8β (0.5 μg/well) in 0.1 M sodium bicarbonate buffer pH 9.6 overnight at 4°C. After blocking the residual free sites with PBS containing 1% bovine serum albumin and 0.1% Tween 20, normal and C8D sera were added at the appropriate dilutions and incubated for 1 h at 37°C and subsequently overnight at 4°C. The binding of the C8 subunits was revealed by the addition of biotin-labeled anti-C8α-γ or anti-C8β antibodies. After an incubation of 2 h at 37°C, the reaction was revealed by the addition of alkaline phosphatase conjugated to streptavidine for 30 min at 37°C, and subsequently of *p*-nitrophenyl-phosphate (Merck, Bracco S. P. A., Milan, Italy) at the concentration of 1 mg/ml in 0.1 M glycine buffer pH 10.4 containing 1 mM $MgCl_2$ as substrate for the enzyme. Reading was performed in a Multiskan 340 apparatus (Flow Laboratories Inc., Milan, Italy) at 405 nm.

Depletion of C8 from human sera by affinity chromatography. Agarose-(α-γ) IgG was prepared by coupling affinity purified goat anti-α-γ IgG to agarose by standard procedures using cyanogen bromide activation (14). This resin recognizes and removes both free α-γ and intact C8 from serum.

Results

Detection of C8α-γ in the C8α-γD sera. Analysis of the three C8α-γD sera by SDS-PAGE under nonreducing conditions followed by immunoblot revealed the presence of C8α-γ in all sera using three different sources of antibodies to C8α-γ, one monoclonal antibody, and two polyclonal antisera. A volume of C8α-γD sera five times higher than that of NHS was required to detect the C8α-γ in the deficient sera, suggesting that the concentration of the subunit in these sera was substantially lower than that observed in NHS. The C8α-γ of the deficient sera was more clearly recognized when the serum precipitate obtained with ammonium sulphate to 37 and 50% saturation was used instead of the whole serum (Fig. 1). This procedure allows analysis of serum samples enriched in their C8 content and that are less contaminated by other serum proteins that contribute to background staining of the nitrocellulose when high serum volumes are used. Like the C8α-γ of NHS, the subunit of the deficient sera exhibited multiple bands when examined under nonreducing conditions. Analysis of the same sera under reducing conditions (results not shown) showed dissociation of the patients C8α-γ into the two constitutive chains α and γ, with molecular weights similar to those of the normal C8 chains.

Quantitation of C8α-γ and C8β by ELISA. The identification of C8α-γ in the three sera lacking hemolytically active C8α-γ prompted us to measure the level of this subunit in the same sera using a polyclonal antiserum specific for C8α-γ and a sensitive ELISA that allowed detection of limited amounts of C8α-γ in NHS. Controls for the specificity of the assay were provided by each serum depleted of C8α-γ by affinity chromatography (see Methods), that gave results essentially similar to those obtained in the absence of serum (blank). Evaluation of the C8α-γD sera by this assay showed C8α-γ levels of ~ 0.5% that of NHS, whereas the amount of C8α-γ in the C8βD sera was closer to that measured in NHS (Fig. 2 *A*).

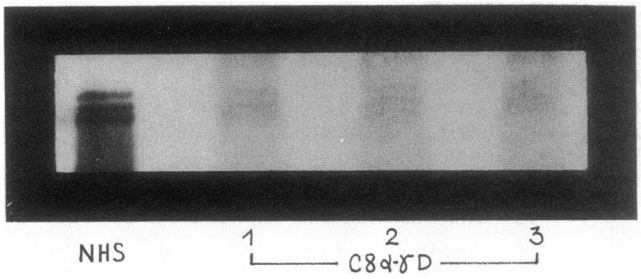

Figure 1. Western blots of three C8α-γD sera and NHS using a polyclonal antiserum to C8α-γ. Each serum sample was treated with ammonium sulphate to 37 and then 50% saturation, and volumes of the precipitates corresponding to 3 and 20 μl of the original serum volume of NHS and the three C8α-γD sera were applied to the gel, respectively.

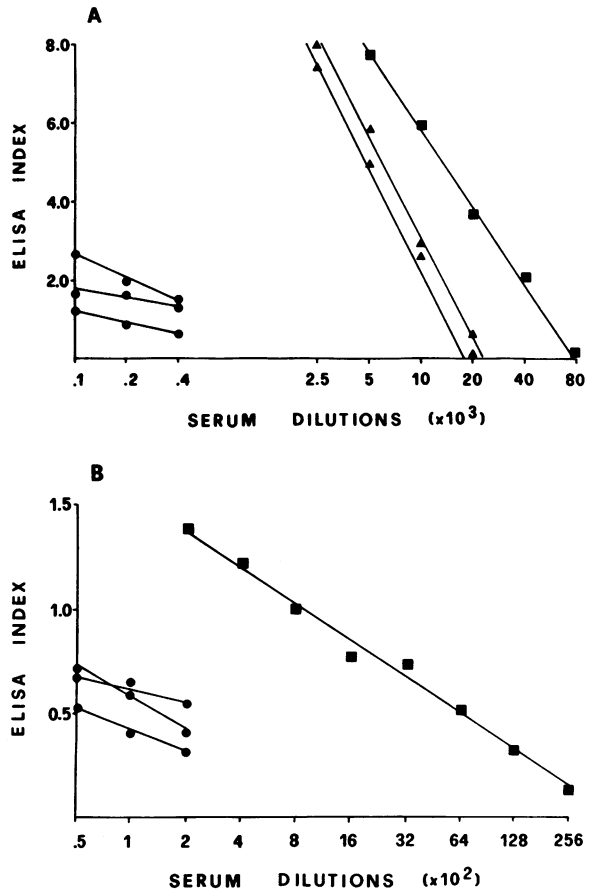

Figure 2. Immunoenzymatic assay of C8α-γ (*A*) and C8β (*B*) in NHS (■ — ■), two C8βD (▲ — ▲), and three C8α-γD sera (● — ●). The results are expressed as ELISA index that is equal to (OD sample—OD blank)/OD blank, where blank indicates the result obtained in the absence of the serum samples.

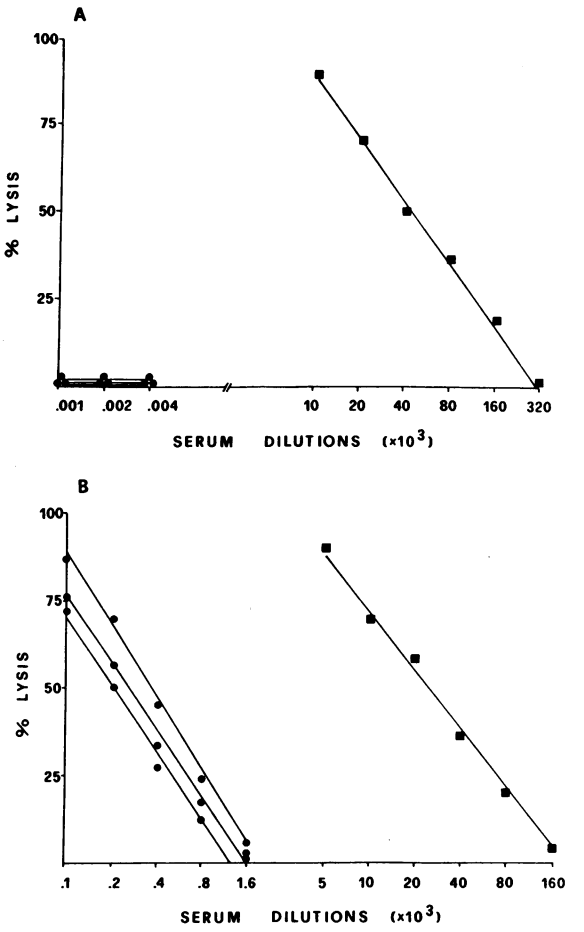

Figure 3. Hemolytic titration of C8α-γ (*A*) and C8β (*B*) in NHS (■ — ■) and three C8α-γD sera (● — ●). The hemolytic system contained 50 μl of EAC1-7 (1.5×10^7) and either C8α-γD or C8βD sera to the final dilution of 1/50 in a total volume of 250 μl.

Since C8β is known to be present in the C8α-γD sera, we wanted to quantitate the amount of C8α-γ relative to that of C8β in these sera next. Employing an ELISA specific for C8β, the level of this subunit in the C8α-γD sera was found to be markedly reduced to ~ 1–3% of that in NHS with a ratio of C8α-γ to C8β varying between 0.2 and 0.5 in the three deficient sera (Fig. 2 *B*). A similar assay performed on the two C8βD sera revealed negligible amounts of C8β (results not shown).

Table I. Comparison of the Hemolytic Activities and the Immunochemical Levels of C8α-γ and C8β Subunits in NHS and C8α-γD Sera

	Hemolytic activity (CH 50 U/ml)		Immunochemical levels (% of NHS)	
	C8α-γ	C8β	C8α-γ	C8β
NHS	160,000	132,000	100	100
C8α-γD1	0	1,750	0.41	2.5
C8α-γD2	0	1,200	0.56	2.1
C8α-γD3	0	1,000	0.50	0.98

The hemolytic activities of C8α-γ and C8β in the C8α-γD sera are differentially related to their immunochemical levels. Having found remarkably low amounts of both C8α-γ and C8β in the three C8α-γD sera by ELISA, it was of importance to examine how these levels related to the functional activity of these subunits in the same sera. Previous work has clearly established that the C8β of the C8α-γD patients is fully active in reconstituting the lytic activity of the C8βD sera (4, 5). Analysis of the C8α-γD sera for C8α-γ (Fig. 3 *A*) and C8β (Fig. 3 *B*) hemolytic activities confirmed the results obtained with the immunoenzymatic assays for C8β but not for C8α-γ. In particular, all three C8α-γD sera had significant C8β activity, but failed to lyse EAC1-8β. The serum of an additional unrelated C8α-γD patient, kindly provided by Dr. H. Jasin (Department of Internal Medicine, University of Texas Southwestern Medical School, Dallas, TX), was also found to have extremely low levels of C8β activity. Unfortunately, this serum was not available in sufficient amounts to allow further investigation. Comparing the results of the ELISA and of functional assays (Table I), it is apparent that the immunochemical levels of C8β in the C8α-γD sera correlates with its functional activity, accounting for 1–3% of the values found in NHS. In contrast, the hemolytic activity of C8α-γ was absent in spite of a

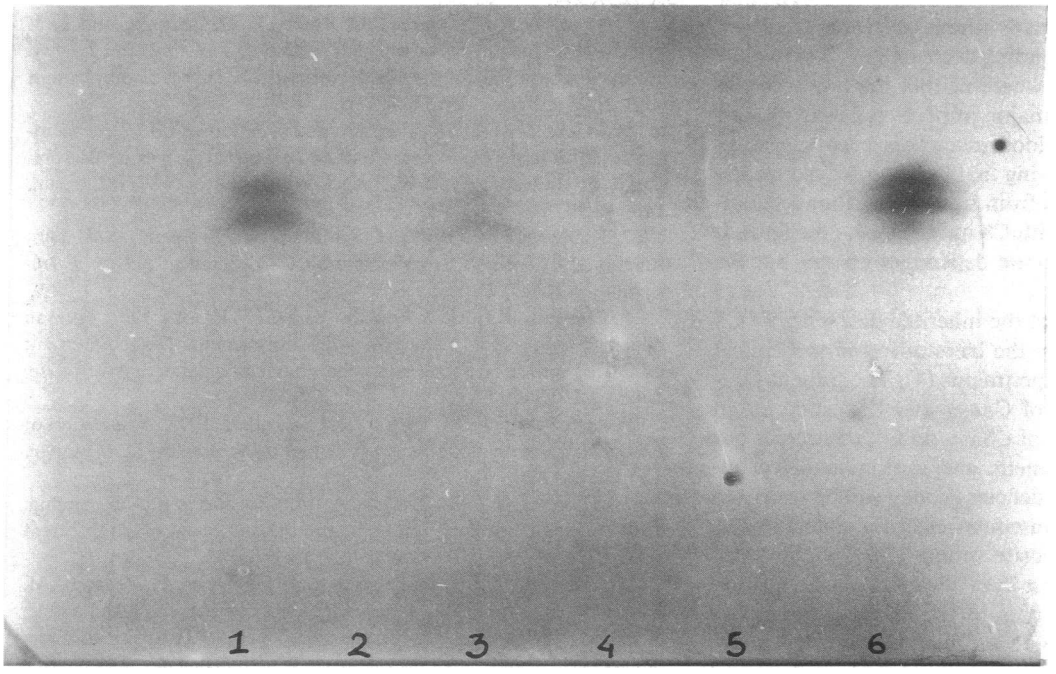

Figure 4. Detection of functional C8α-γ subunit by hemolytic overlay. 1 μl of NHS (lane *6*) and 6 μl of two C8βD sera (lanes *1* and *3*) and of three C8α-γD sera (lanes *2, 4,* and *5*) were electrophoresed by SDS-PAGE and the hemolytic bands were revealed as described in Methods.

detectable immunochemical level, suggesting that C8α-γ in these sera is hemolytically inactive.

To obtain further evidence in favor of a dysfunctional C8α-γ molecule being present in the C8α-γD sera, an additional approach was followed based on the hemolytic detection of C8α-γ after SDS-PAGE. For this purpose, the sera were electrophoresed in duplicate on the same gel, and at the end of the run, one-half of the gel was processed for Western blotting while the second half was used for the hemolytic overlay after removal of SDS. Under these conditions, the C8α-γ of both NHS and C8βD sera exhibited activity, as indicated by the appearance of lytic bands at the position of C8α-γ migration (Fig. 4). No bands were detected in lanes loaded with the three C8α-γD sera, although distinct C8α-γ bands appeared in the corresponding lanes of the gel after immunoblotting (results not shown).

Discussion

Patients with C8α-γ deficiency represent a minority of the whole group of C8D individuals and can be identified by the presence of hemolytically active and immunochemically recognizable C8β in their sera (2). The lack of functional activity in these sera has been attributed to a selective defect of C8α-γ that could not be detected either functionally or immunochemically. Although this conclusion remains basically correct, the present data indicate that the nature of the C8 defect in these sera is more complex than hitherto recognized.

An important finding of this study was the detection of C8α-γ in the deficient sera by Western blot analysis of the whole serum using a monoclonal antibody and two polyclonal antisera to C8α-γ. The higher sensitivity of both the assay system employed in this investigation and the use of antibodies specific for C8α-γ, possibly directed to additional epitopes not recognized by antisera to whole C8, may have contributed to reveal previously undetected C8α-γ. A similar situation has been described by Tschopp et al. (7) in two C8βD sera in which C8β was detected only by antisera specific for C8β but not by those to whole C8. Quantitation of C8α-γ by ELISA confirmed the presence of this subunit in the C8α-γD sera, albeit in a limited amount that did not exceed 2% of the level observed in NHS. The fact that the antiserum used for the ELISA detected only C8α-γ in NHS by Western blot proves the specificity of the assay for C8α-γ. This was further substantiated by the finding that depletion of the residual C8α-γ by affinity chromatography resulted in C8α-γD sera values similar to those of the controls in the absence of serum.

The low levels of C8α-γ found in the C8α-γD sera do not correlate with the lack of hemolytic activity in these sera. NHS can in fact be diluted to contain an amount of C8α-γ equal to that of the C8α-γD sera and still induce complete lysis in a C8α-γ-dependent lytic assay. This assay is in fact exquisitely sensitive and reveals functional C8α-γ in NHS up to a dilution of 1 to 100,000. The suggestion can therefore be made that the residual C8α-γ in the deficient sera is dysfunctional. This is also proved by the inability of the subunit in the C8α-γD sera to resume lytic activity after SDS-PAGE in an overlay system, in spite of the fact that C8α-γ can be detected immunochemically by Western blot.

Discovery of a combined deficiency of α-γ and β in C8α-γ patients is unexpected in view of the fact that the amount of C8β in these sera is sufficient to reconstitute the lytic activity of C8βD sera (4), and also that in C8βD sera α-γ is frequently present at near normal levels. If inherited C8 deficiency manifests itself as a defect in either α-γ or β, then one would also expect to find near normal levels of β in C8α-γD. Results of this study clearly indicate that this is not the case. One explanation may be that in C8α-γD sera there are molecular defects in both α-γ and β such that levels of each are reduced. This seems unlikely however, because only α-γ is functionally abnormal. An alternative explanation is that β depends on association with normal α-γ for proper biosynthesis and secretion

or for stability in the circulation. Synthesis of an abnormal α-γ would then produce a corresponding decrease in β. This would contrast with C8β deficiency, where neither the level nor the functional state of β have a major impact on levels of α-γ. Stability of free α-γ in the bloodstream has been suggested from biosynthetic studies showing that hepatocytes secrete excess α-γ relative to β (14), and from studies that found significant levels of free α-γ along with C8 in NHS (19, 20). Thus, it may be that serum levels of β are dependent on α-γ but the converse is not necessarily true.

We originally proposed that the inherited deficiency of C8 be divided into two groups on the basis of immunochemical analysis and reconstitution experiments (4). The present findings of combined deficiency of C8α-γ and C8β subunits in patients with inherited defect of C8α-γ do not contradict our previous observation. The genetic and molecular defect responsible for each type of C8 deficiency may still be restricted to either α-γ or β, but the consequences of these deficiencies differ with regard to the opposite subunit. In the combined deficiency for instance, a defect in α-γ would have an adverse effect on serum levels of β. At present, the nature of the molecular defects responsible for C8 deficiency is unknown. A major deletion of the β gene has been excluded based on studies of genomic DNA from patients with C8β deficiency (21), while for the combined deficiency the defect may be in α or γ. A major gene deletion in α seems unlikely in view of the close physical linkage between α and β on chromosome 1 and the expression of functional β in the C8α-γD (22). The exact nature of the α-γ defect must await further characterization of α and γ genomic structures.

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