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REDUCED C8β MESSENGER RNA EXPRESSION IN FAMILIES WITH HEREDITARY C8β DEFICIENCY[1]

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Individuals with functional C8β deficiency are at increased risk for systemic neisserial infections. Studies by others have shown that the structural gene for this protein appears intact in deficient individuals. We studied affected individuals from 10 unrelated families to determine the basis for their defect. Using chain-specific antisera, C8β was undetectable on immunoblots of their sera. The polymerase chain reaction was used to probe cDNA synthesized from RNA isolated from human liver cells, HepG2 cells, peripheral blood monocytes, and fibroblasts to identify a readily available cell source expressing C8β message. Cells from each of these sources expressed C8β message. The identity of the amplified product was confirmed and this approach was used to probe cDNA synthesized from RNA harvested from monocytes or fibroblasts obtained from two unrelated families with C8β deficiency. C8β mRNA was readily detectable in C8β sufficient and heterozygous family members but required Southern blotting and hybridization to the ^{32}P-labeled C8β probe for detection in the homozygous deficient probands. These results suggest that C8β-deficient individuals produce less C8β-specific mRNA than do normals and that the underlying basis for this deficiency is an abnormality in intracellular events that precede secretion.

C8 is one of the late-acting components of the C cascade. Like the other terminal C components it plays an essential role in the formation of the membrane attack complex responsible for C-dependent membrane damage and bactericidal activity. However, C8 is unique in that it consists of two functional subunits, α-γ and β, composed of three polypeptide chains (1, 2), each of which is encoded by a separate structural gene (3–6). The α-chain has a molecular mass of 64 kDa and is linked to the 22-kDa γ-chain by a single disulfide bond. In contrast, the 64-kDa β-chain is linked to the α-γ subunit by noncovalent interactions. Functional activity of the molecule seems to reside in α and β, since γ does not appear necessary for C8 activity in vitro (7).

Two hereditary C8 deficiency states have been described; functional α-γ subunit deficiency and the more common functional β subunit deficiency (8, 9). These deficiency states segregate along racial origins (10). Irrespective of the subunit involved, patients with C8 deficiency have increased susceptibility to neisserial infections (10).

Individuals with a functional deficiency of the β-subunit appear to have an intact β structural gene based on RFLP patterns on Southern blots (11). One report suggests the possibility that C8β functionally deficient sera may contain immunoreactive C8β when examined with subunit-specific antisera (12). Other studies have failed to detect C8β in functionally deficient individuals using antisera to the intact C8 molecule (13).

The present report details our observations of C8β protein and RNA in 10 unrelated C8β-deficient families. Our findings indicate that monocytes and fibroblasts from homozygous C8β-deficient individuals contain reduced amounts of C8β mRNA and suggest that a transcriptional defect is the likely basis for this deficiency.

MATERIALS AND METHODS

Serum samples. Sera from 20 men and women were collected and pooled for use as a NHS[3] control in some studies. NHS immunodepleted of C8 was prepared as described previously (14). Serum was also obtained from 10 unrelated individuals with proven functional C8β deficiency. All sera were stored at −75°C.

C8β-deficient patients and families. The individual C8β-deficient persons whose sera were examined by immunoblotting have been reported previously (patients 3, 17, 28, and 30 in Table 2,I of ref. 10) or will be shortly (patients 51–53, 58, 66 ,67 in Table 3,J of ref. 15). Monocytes and fibroblasts were obtained from two C8β-deficient families. The first (patient 52, Table 3,J of ref. 15) consisted of the deficient individual, her heterozygous parents, normal brother, and husband. Individuals from the second family (9) who were studied included the deficient proband, his heterozygous parents, and a normal brother.

Antiserum. Rabbit antiserum to whole C8 was obtained from Calbiochem (San Diego, CA). To generate subunit-specific antisera, purified whole C8 (Quidel, San Diego, CA) was electrophoresed on a nonreducing SDS polyacrylamide gel (16) and a vertical lane in the margin of the gel stained with Coomassie blue to determine the location of the α-γ and β-subunits. The corresponding horizontal regions of the gel were excised and the proteins electroeluted (Elutrap, Schleicher and Schuell, Keene, NH). Antiserum was obtained by immunizing rabbits s.c. with 100 μg of C8β in CFA followed by two monthly injections of 50 μg emulsified in IFA.

Immunoblotting of proteins. Serum samples (4 μl) were heated to 95°C for 3 min and separated by SDS-PAGE under nonreducing conditions. Proteins were electrophoretically transferred to nitrocellulose (Schleicher and Schuell) as described by Towbin (17), and the membranes blocked with 5% BSA in PBS. The appropriate unmodified rabbit antisera were added (anti-C8, 1:500, anti-C8β 1:50 dilution in PBS) and incubated at 4°C overnight. The membranes were washed three times with 1% BSA in PBS and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (Sigma

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[3] Abbreviations used in this paper: NHS, normal human serum; PCR, polymerase chain reaction.

Chemical Co., St. Louis, MO). Membranes were washed three times with 0.1% Tween 20 in PBS followed by veronal acetate buffer (pH 9.6) and developed using nitroblue tetrazolium chloride (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as substrates in veronal acetate buffer.

Cell separation and culture. Peripheral blood monocytes were obtained from whole blood using Histopaque (Sigma) for initial separation and were allowed to adhere to culture plates (Flow Laboratories, Inc., McLean, VA). Cells were cultured for 4 to 36 h in Medium 199 with 13% autologous serum. Plates were washed three times to remove nonadherent cells. HepG2 cells (American Type Tissue Culture Collection, Rockville, MD) were cultured in Eagle's MEM containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine. Human fibroblasts were isolated from skin biopsies from normal or C8-deficient individuals and cultured in α-MEM supplemented with 10% FCS, penicillin, and streptomycin.

RNA. Total cellular RNA was extracted from cell monolayers by guanidinium thiocyanate (Boehringer Mannheim Biochemicals, Indianapolis, IN) lysis followed by cesium chloride density gradient ultracentrifugation as described by Chirgwin et al. (18). In some cases, poly-A RNA was isolated directly from cultured cells utilizing oligo-dT cellulose affinity chromatography (Invitrogen, San Diego, CA). Poly-A RNA from human liver was kindly provided by Dr. Harvey Colten, Washington University, St. Louis, MO.

Reverse transcription. Reverse transcription of RNA (1 μg/10 μl) to generate cDNA was performed by incubation of total cellular RNA or poly-A RNA at 37°C for 60 min in a mixture of 100 U Moloney murine leukemia virus-reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), 0.2 μg of oligo-dT primer (Pharmacia, Inc., Piscataway, NJ), 3.0 mM $MgCl_2$, 10 mM Tris HCl (pH 8.3), 75 mM KCl, 1 μg of acetylated BSA, 0.5 mM dNTP, and 5 U of RNasin (Promega Corp., Madison, WI) in a final volume of 10 μl (19).

Polymerase chain reaction. Oligonucleotide primer pairs specifying regions of the 5′ and 3′ ends of C8β mRNA were selected based on the published cDNA sequence (3, 4) and synthesized at the University of Iowa DNA core facility. Most of the amplification reactions employed the 3′ primer pair since oligo-dT-based cDNA synthesis is more efficient at this end of the mRNA molecule. These primers were 24 and 22 bp in length and defined a 169-bp region extending from 1546 to 1714 in C8β mRNA (3). This fragment contains an internal *Bam*HI restriction enzyme site, cutting at which yields 135- and 34-bp fragments. The 5′ primers were 23 and 24 bp in length and defined a 308-bp fragment extending from 217 to 524 in C8β mRNA (3). In some experiments a set of 20 bp primers (5′ primer beginning at nucleotide 1514 and 3′ primer ending at nucleotide 1904 of β-actin genomic DNA (20)) delineating a 271-bp fragment in mature β-actin mRNA was amplified as a transcriptional control. A 1- to 2-μl sample of the cDNA mixture was mixed with 1 U of Taq DNA polymerase (Cetus Corp., Emeryville, CA), 50 pmol of the specific primers in 20 mM Tris HCl buffer, (pH 8.3), containing 0.2 mM dNTP, 2.0 mM $MgCl_2$, 50 mM KCl, and 5 μg of acetylated BSA, in a final volume of 50 μl. The reaction mixture was overlaid with light mineral oil and amplified by the PCR in a repeated three temperature cycle in a programmable heating block (Perkin Elmer/Cetus thermocycler, Norwalk, CT). Denaturation was promoted at 94°C for 30 to 60 s, annealing at 55°C for 30 to 60 s and extension at 72°C for 1.5 to 2 min depending on the length of the sequences to be amplified; 30 to 40 cycles were used for amplification. Reaction mixtures lacking RNA or reverse transcriptase were included in each series of experiments as negative controls and to assess the possibility of cross-contamination.

Agarose gel electrophoresis. Ten-microliter aliquots of PCR mixtures were electrophoresed in a 100-V constant voltage field in an agarose gel consisting of a mixture of 3% NuSieve GTG/1% Seakem ME agaroses (FMC Bioproducts, Rockland, ME). Gels were stained with 1 μg/ml ethidium bromide for 10 min, destained in H_2O for 1 h, visualized under UV light, and photographed.

Southern blot analysis. DNA separated on agarose gels was alkaline denatured and transferred (21) to Nytran paper (Schleicher and Schuell). Nytran blots were hybridized to a random primed (Boehringer Mannheim Biochemicals) ^{32}P-labeled probe derived from full length C8β cDNA (3) (kindly provided by Dr. James Sodetz, University of South Carolina), washed using high stringency conditions, exposed to Kodak XAR film at −80°C using an intensifying screen, and developed 24 h later.

Restriction enzyme analysis. The PCR-amplified products were extracted in phenol:chloroform:isoamyl:alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) before ethanol precipitation. The precipitate was digested with *Bam*HI and the products of the digest separated on a 4% agarose gel.

RESULTS

Immunoblots of normal and C8β-deficient serum. Immunoblots of serum samples run under nonreducing conditions (Fig. 1) readily demonstrated C8β in NHS. The specificity of this detection system was confirmed by the marked reduction in immunoreactive material in NHS immunodepleted of C8. In contrast, C8β was not detected at all in the sera of the six unrelated functionally C8β-deficient individuals. Identical results were obtained with the sera of four additional unrelated patients (data not shown). Based on the concentration of C8 in NHS (50 ng/μl), the proportionate amount of C8β in C8 (43%), and titration to determine the smallest volume (0.04 μl) of NHS in which C8β could be detected reliably on immunoblots, the sensitivity of this procedure for detecting C8β was approximately 0.84 ng. Thus the concentration of C8β in the deficient sera must be less than 0.21 ng/μl or less than 1% of that present in NHS.

Cell sources expressing C8. To further elucidate the basis for C8β deficiency it was necessary to identify a readily accessible cell source that produces C8. Indirect evidence suggested that monocytes, which synthesize some early C components (22), might synthesize low levels of the terminal C components as well (23). However, we were unable to detect C8 activity in 50-fold concentrated, normal monocyte-conditioned media using a functional assay capable of detecting as little as 50 pg C8/ml. Northern blots of total monocyte RNA failed to demonstrate C8β message (data not shown).

Because we were concerned that these assays might be insufficiently sensitive, we used PCR to detect low levels of C8β specific mRNA (Fig. 2). The predicted 169-bp product was generated when RNA from normal liver, which is known to synthesize C8, was used for cDNA synthesis before the amplification reaction. An identical sized fragment was also detected when two different concentrations of peripheral blood monocyte RNA were employed in the reaction. In contrast, this product was not observed in ethidium bromide-stained gels of reaction mixtures containing fibroblast RNA.

The amplified products from this gel were transferred to Nytran paper and hybridized with a ^{32}P-labeled full length C8β cDNA probe (Fig. 3). Using this method of detection, specific hybridization was evident with each of the sources of RNA-derived cDNA. The identity of the amplified product was confirmed by restriction enzyme digestion with *Bam*HI (Fig. 4) yielding the 135-bp and 34-bp fragments predicted from the reported C8β sequence (3, 4). Residual uncut 169 bp is evident in this experiment because the *Bam*HI concentration selected for digestion was based on the random distribution of these restriction

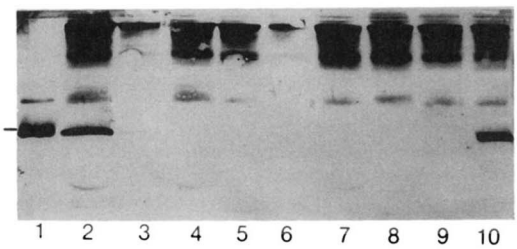

Figure 1. Detection of C8β in normal and C8β-deficient sera by immunoblotting. *Lane 1* contains purified C8 (0.5 μg). The remaining lanes each contain 4 μl of serum; *lanes 2* and *10*, pooled NHS; *lane 3*, NHS immunodepleted of C8; *lanes 4* to *9* sera from six unrelated C8β-deficient individuals.

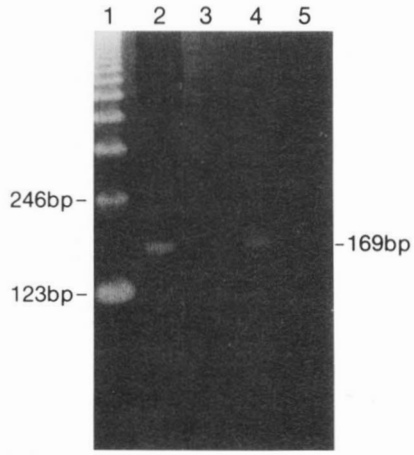

Figure 2. PCR amplification of C8β cDNA synthesized from different cellular sources of RNA. *Lane 1*, molecular size standards; *lane 2*, 1 μg of normal human liver poly-A RNA; *lanes 3* and *4*, 0.5 and 1 μg, respectively, of normal monocyte RNA; *lane 5*, 1 μg of fibroblast RNA.

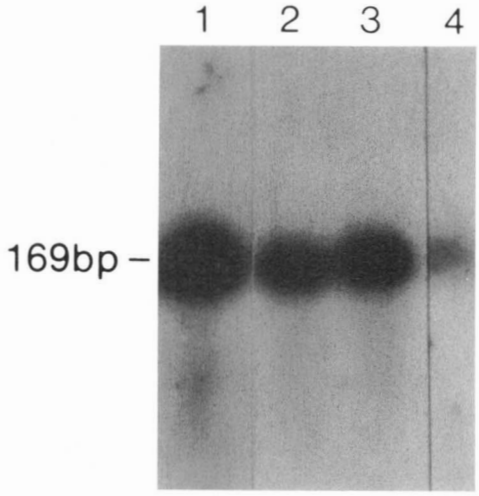

Figure 3. Hybridization of full length ^{32}P-labeled C8β cDNA to a Southern blot of the amplified C8β product shown in Figure 2. *Lane 1*, 1 μg of normal human liver poly-A RNA; *lanes 2* and *3*, 0.5 and 1 μg, respectively, of normal monocyte RNA; *lane 4*, 1 μg of fibroblast RNA.

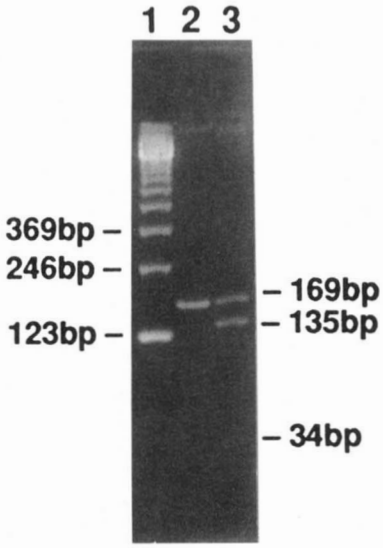

Figure 4. *Bam*HI restriction enzyme mapping of amplified C8β product. *Lane 1*, molecular size standards; *lane 2*, undigested PCR product; *lane 3*, *Bam*HI digested product.

sites in nonselected high-molecular mass DNA rather than on a single site every 169 bp in the selected cDNA that results from the PCR. In addition the identity of the amplification product was verified by direct sequencing of a 22-bp internal segment (data not shown).

Detection of C8β mRNA in deficient individuals. Using this approach we examined RNA from peripheral blood monocytes from the members of a family with functional C8β deficiency. By ethidium bromide staining, the 169-bp C8β amplification product was readily observed with cDNA derived from monocyte RNA from each of the proband's obligately heterozygous parents and her husband. In contrast, this product was not detected when RNA harvested from the monocytes of the C8β-deficient proband was used for cDNA synthesis (Fig. 5*A*). Parallel amplifications using oligonucleotide primers specifying a 271-bp segment of the cytoskeleton protein β actin (19) confirmed that equivalent cDNA synthesis occurred in each of the reverse transcriptase reactions (Fig. 5*B*). This result indicates that the failure to detect C8β specific product in the deficient individual was not a consequence of faulty cDNA synthesis in that reaction. In separate experiments employing Southern blot transfer and hybridization to the ^{32}P-labeled full-length C8β probe, the 169-bp C8β amplification product was readily identified in each of the samples, regardless of the C8β status of the individual (Fig. 6). These results indicate that a low

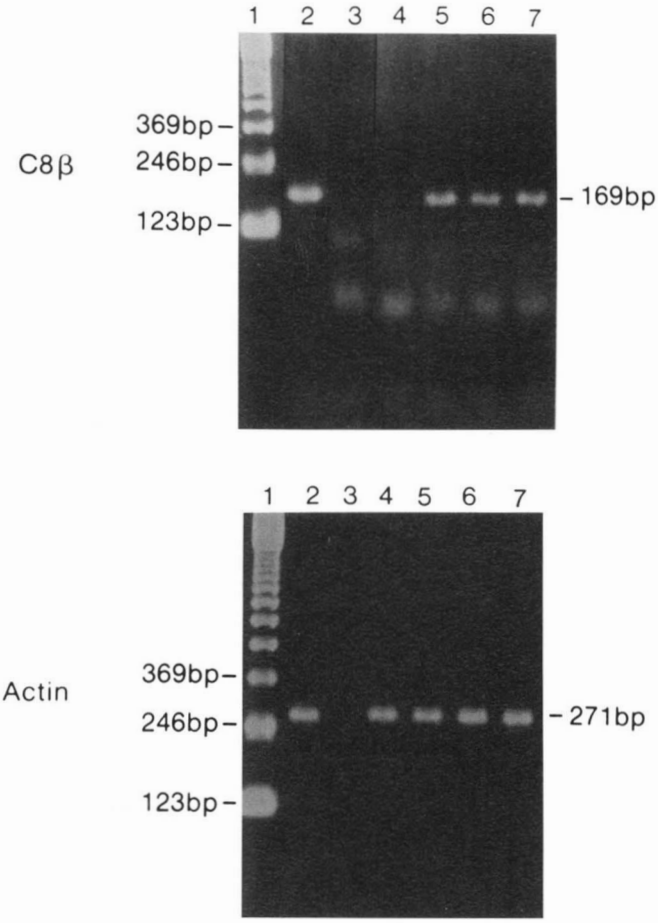

Figure 5. Detection of C8β specific cDNA derived from monocyte RNA from a C8β-deficient family. *Upper panel*, C8β amplification. *Lane 1*, molecular size standards; *lane 2*, HepG2 cDNA; *lane 3*, reverse transcriptase negative control; *lanes 4* to *7*, monocyte cDNA from the proband, mother, father, and husband, respectively. *Lower panel*, β-actin amplification of the same reverse transcriptase reactions shown in the *upper panel*.

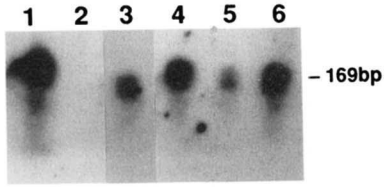

Figure 6. Hybridization of full length ^{32}P-labeled C8β cDNA to a Southern blot of amplified monocyte C8β cDNA from a family with C8β deficiency. *Lane 1*, HepG2 cDNA; *lane 2*, reverse transcriptase negative control; *lanes 3* to *6*, monocyte cDNA from the proband, brother, father, and mother, respectively. This is the same family depicted in Figure 5.

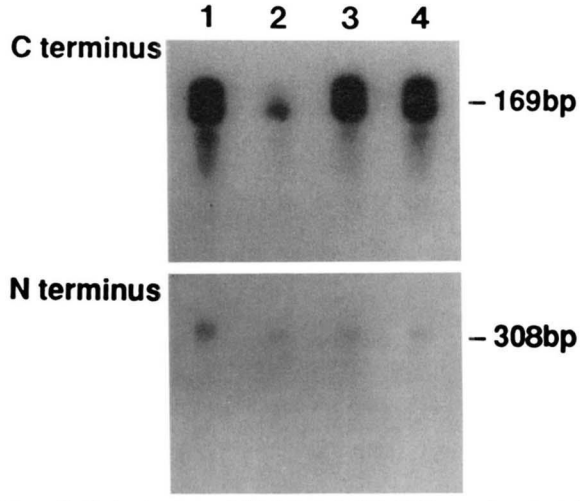

Figure 7. Hybridization of ^{32}P-labeled C8β cDNA to a Southern blot of amplified monocyte C8β cDNA from a second family with C8β deficiency. *Upper panel*, amplification using a C-terminus primer pair. *Lower panel*, amplification using an N-terminus primer pair. *Lanes 1* to *4*, cDNA from the proband, mother, father, and brother, respectively.

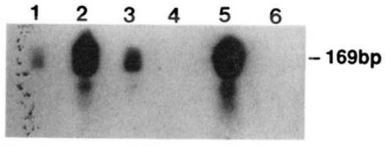

Figure 8. Hybridization of ^{32}P-labeled C8β cDNA to amplified fibroblast cDNA from C8β-sufficient and -deficient individuals. *Lanes 1* and *2*, fibroblast cDNA from a C8β-deficient and -sufficient individual, respectively; *lane 3*, normal monocyte cDNA; *lane 4*, reverse transcriptase negative control; *lane 5*, Hep G2 cDNA; *lane 6*, PCR negative control.

level of C8β specific mRNA was present in the monocytes of this C8β functionally deficient individual. Monocytes from a second unrelated C8β functionally deficient individual and his parents were studied (Fig. 7). Again the 169-bp product could be identified after amplification of cDNA derived from the proband as well as from his obligately heterozygous parents using Southern blotting and hybridization to the ^{32}P-labeled C8β cDNA probe. A separate amplification, using a second set of primers specifying a 308-bp segment of the N-terminus region of the C8β mRNA, demonstrated the presence of the predicted product in all of the reactions containing cDNA derived from the RNA isolated from each of these individuals' monocytes. The presence of C8β mRNA in fibroblasts from the deficient proband in the second family was also demonstrated by Southern blotting of PCR products and hybridization with the ^{32}P-labeled C8β probe (Fig. 8).

The exquisite sensitivity of the PCR to detect minute quantities of target DNA not only makes it a powerful tool for investigations like this but also makes it vulnerable to false-positive results due to contaminating DNA. Evidence that contamination does not account for the results presented here includes: 1) a consistent pattern of results in two unrelated families in multiple experiments employing RNA from two different cell types; and 2) the absence of a detectable amplification product in ethidium bromide-stained agarose gels or Southern blots hybridized to the ^{32}P-labeled C8β probe for reactions in which either the reverse transcriptase was omitted or genomic DNA (data not shown) was added.

DISCUSSION

C8β deficiency is an inherited autosomal recessive disorder that is most frequently detected as a consequence of increased susceptibility to neisserial infections. The C8β gene is located on chromosome 1 (24, 25) and appears intact in C8β-deficient individuals as judged by Southern blot analysis of RFLP (11).

In the studies reported here we were unable to detect immunoreactive C8β in the sera of 10 unrelated C8β functionally deficient individuals using antiserum raised against purified C8β. In contrast, C8β was readily detected in NHS in 0.01 volume of C8β-deficient sera applied to the gel. These findings suggest that the basis for this deficiency in these families is not the secretion of an abnormal C8β molecule. These results are similar to the preliminary findings of others (13) using antisera specific for intact C8, but differ from those of Tschopp et al. (12). The latter investigators described two unrelated C8β-deficient individuals whose sera, when examined by SDS-PAGE and immunoblotting, contained a protein that reacted with C8β specific antiserum and had an apparently normal molecular size. However, the C8β immunoreactive products detected after SV8 protease digestion of normal and C8β-deficient serum differed in molecular mass. These findings raise the possibility that there may be a heterogeneous basis for C8β deficiency. The fact that the C8A and B loci are tightly linked on the short arm of chromosome 1 (24–26), coupled with the recent demonstration that C8β deficiency occurs in association with a limited number of C8α polymorphisms (13, 26), is also consistent with a limited number of molecular defects causing this deficiency.

A major limitation in addressing the molecular basis for the terminal complement component deficiency states has been the absence of a readily accessible cell source that expresses the products of the respective structural genes. On the basis of functional studies, previous reports (22, 23) suggested that peripheral blood monocytes may synthesize these C components. A significant result of our studies was to definitively establish that cultured peripheral blood monocytes and fibroblasts express C8β message as shown by the presence of the C8β-specific 169-bp amplification product in ethidium bromide-stained gels and Southern blot hybridization to a full length C8β cDNA probe. We anticipate that the capability of establishing and storing long-term primary fibroblast cultures from deficient individuals will be an invaluable asset in the study of these disorders. The finding that the C8β gene is grossly intact in deficient individuals (11) and our results demonstrating the presence of C8β-specific mRNA in both monocytes and fibroblasts from C8β-sufficient and -deficient individuals from two unrelated C8β-deficient families indicate that neither a major gene deletion nor the absence of transcription is the basis for

the deficiency state. The fact that both 5′ and 3′ segments of cDNA derived from C8β mRNA could be amplified suggests that a truncated message as the cause of C8β deficiency is less likely. However, these results do not exclude the possibility of small deletions or insertions within the body of the mRNA. Such defects could arise as a result of aberrant splicing and give rise to an abnormal-sized message. The lack of sensitivity of Northern blots for the detection of C8β mRNA in the RNA from cell sources available even from normal individuals and our inability to amplify the entire C8β message preclude an assessment of these possibilities at the present time.

The fact that Southern blotting and hybridization to a labeled probe were necessary to detect the C8β-specific amplification product in deficient but not sufficient individuals suggests that the relative amount of C8β-specific mRNA is lower in deficient persons. This finding together with the failure to detect even small amounts of C8β in the serum of deficient individuals suggest that a transcriptional abnormality may contribute to this defect. Alternatively, decreased amounts of specific mRNA could arise as a consequence of an abnormality in the promoter portion of the gene, although in this case the mRNA produced should be normal and result in the synthesis of active C8β. Our inability to detect C8β protein in deficient individuals suggests that this possibility is less likely. Finally, an abnormality affecting message stability could account for the low levels of C8β mRNA in deficient individuals. Such a defect might lead to the production of low levels of an abnormal C8β subunit that failed to associate with C8α-γ or was susceptible to proteolytic degradation thereby accounting for the absence of detectable C8β in serum. The development of techniques to quantitate C8β mRNA should help to assess these possibilities.

In conclusion, studies involving immunoprecipitation of intrinsically labeled protein are necessary to exclude a defect in secretion or secretion of an unstable molecule. Determination of the sequence of the C8β mRNA in deficient individuals will help to distinguish among these possibilities and further characterize the basis for the defect. Once a basis for the defect has been established, additional studies will be necessary to assess its prevalence among all individuals with C8β deficiency to address the possibility of a heterogeneous basis for the defect. Given the high degree of homology among C8β, C8α, and the other terminal C components (27–29), such studies may shed light on the molecular basis for inherited deficiencies of these other molecules as well.

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