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Hypomorphic *C4B*15* variant of the fourth component of complement

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We report a rare 'hypomorphic' C4 allotype detected during routine screening in controls for the Rogers:1 epitope. *C4B*15* was distinguished by having only faint staining when using polyclonal anti-C4 antibody on agarose immunoelectrophoresis (e.g. hypomorphic), having relatively weak hemolytic activity but being strongly reactive with monoclonal antibody to Rodgers 1. TaqI restriction fragment length polymorphism (RFLP) demonstrated that *C4B*15* segregated with 7 kb and 5.4 kb C4 gene fragments and with the haplotype *HLA-A2,C-, B50,BW6,DR7,DQ2,DR52,SO7C2(1,15)*. The 5.4-kb fragment was more intense than the 7.0-kb fragment, suggesting duplication of the 5.4-kb fragment. This hypomorphic C4 allotype (genotype frequency = 0.0088) has diminished expression of C4 epitopes commonly recognized by polyclonal anti-C4 and may be missed by standard phenotyping methods.

C4 allotype; Complement; Polymorphism; Restriction fragment length polymorphism; Immunogenetics

1. INTRODUCTION

Human C4 protein is synthesized as a single pro-C4 molecule and secreted into serum as a three-chain protein. C4 is normally encoded by one of two loci (*C4A* or *C4B*) lying within the major histocompatibility complex (MHC) between the *HLA-B* and the *HLA-D* complex on chromosome 6 [1]. C4A and C4B isotypes differ in several ways but, with notable exceptions [2], C4A proteins carry one or more Rodgers epitopes, while C4B proteins carry one or more Chido epitopes.

A rare C4 allotype has been detected which reacted strongly with a monoclonal antibody to Rodgers:1 (Rg:1) but was very faint when using polyclonal anti-C4. Using a combination of protein and DNA analyses, we have characterized both this C4 gene and its protein product.

2. MATERIALS AND METHODS

2.1. *Agarose gel typing of C4*

Disodium (ethylenedinitrilo) tetraacetic acid (EDTA) plasma was subjected to electrophoresis following incubation with carboxypeptidase B and with neuraminidase [3]. Goat polyclonal anti-C4 (Atlantic Antibodies, Scarborough, ME) was then applied to the gel for immunofixation. The pressed gel was stained with Coomassie Blue in acetic acid after washing in saline.

2.2. *Anti-Rodgers immunofixation*

Agarose electrophoresis was first performed as described above and proteins were then transferred to a nylon membrane. The membrane was incubated with mouse monoclonal anti-Rodgers (MAB anti-Rg:1) antibody in a concentration of 1:250 at room temperature for 2 h (kindly provided by C. Giles, London, England). Following washing of the membrane (Phosphate-buffered saline (PBS) and polyoxyethylenesorbitan monolaurate (Tween 20) incubation with goat peroxidase-conjugated anti-mouse IgG (Sigma A:4416) at a dilution of 1:300, overnight at 4°C was performed. PBS, hydrogen peroxide, and 3',3-diaminobenzidine tetrahydrochloride (15 ml, 7.5 ml, 7.5 μg, respectively) were used to develop the peroxidase-stained bands of positive allotypes.

2.3. *Western blot*

Human C4 was precipitated from EDTA plasma with goat anti-human C4 antibody [4]. The precipitated gel was washed in 0.25 M EDTA (pH 7.5) and disintegrated in a sample buffer containing 10% 2-mercaptoethanol. Samples were run on a 7.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) at 450 V for 2 h as a vertical gel and cooled to 10°C. Proteins in the gel were blotted to a nylon membrane at 30 V in a 20% methanol solution containing 25 mM Tris(hydroxymethyl)aminomethane and 192 mM glycine overnight at 10°C. The dried membrane was incubated with monoclonal anti-Rg:1 in a 1:20 concentration for 2 h at room temperature. The membrane was then washed in PBS with Tween 20 and incubated with peroxidase-conjugated goat anti-mouse IgG overnight at 4°C. The peroxidase was developed as described above. Using SDS-PAGE, one can separate the α-chains of C4A (96 kDa) from C4B (94 kDa).

2.4. *DNA analysis*

DNA was isolated from EDTA-treated whole blood using standard methodology. DNA was digested with TaqI according to manufacturer's suggestion, electrophoresed in an 0.8% agarose gel overnight, and transferred to a nylon membrane under alkaline conditions. The membranes were hybridized with a ^{32}P-radiolabelled 0.6 kb fragment of *pAT-A*, a 5'-end-oriented fragment. With TaqI restriction enzyme digestion, C4A genes yield a 7 kb fragment, C4B genes yield 6.0 and 5.4 kb fragments [3]. A C4A gene deletion on the haplotype *HLA-A1,B8,Cw7,DR3,DQ,DR/SC01* will characteristically yield a unique 6.4 kb TaqI fragment, although this C4 deletion is also found on other haplotypes.

2.5. *HLA typing*

HLA typing was performed using standard techniques with an-

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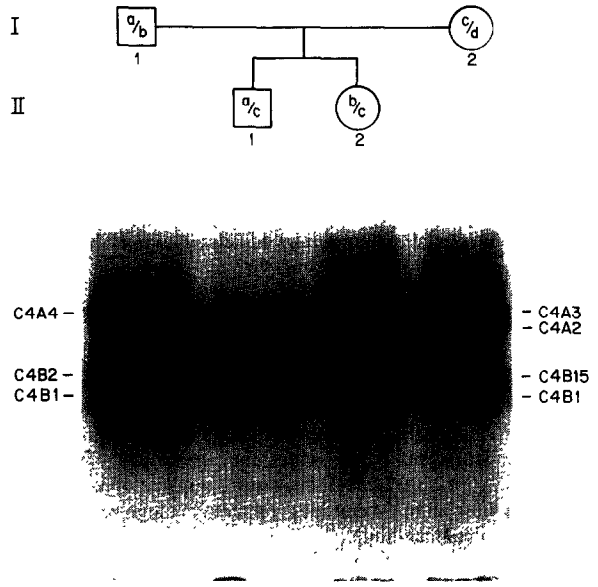

Fig.1. The C4 phenotype of pedigree Ju. The C4 phenotype by immunodiffusion assay demonstrates that the proband (II-1) has the faint allotype (*C4B*15*) which is more clearly seen in the mother's specimen (I-2) but is obscured by *C4B*2* in the sibling (II-2).

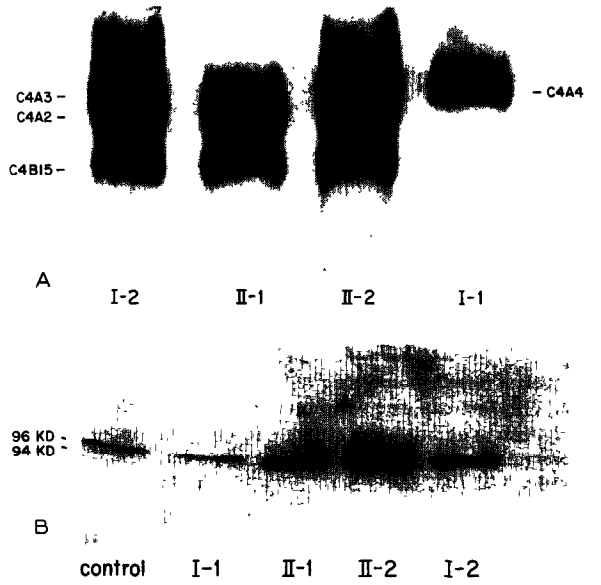

Fig.2. Immunoblot and SDS-PAGE. (A) Rg:1 immunoblot: following transfer of proteins to a nylon membrane, anti-Rg:1 shows the *C4B*15* allotype in the mother (I-2) and both siblings (II-1, II-2). (B) SDS-PAGE: following immunoprecipitation, washing and reduction, C4 α-chains were separated by vertical gel SDS-PAGE electrophoresis and Western blotting. The control specimen and the father had a single α-chain of 96 kDa which reacted with MAB anti-Rg:1. The mother and both children have two α-chains reactive with anti-Rg:1, confirming the presence of a Rg:1-positive C4B allotype in these individuals. The solitary band above the 96 kDa band in subject II-2 is an artifact.

tiserum from the NIH serum bank and was compared to a 60-cell panel of known specificities.

2.6. *Pedigree Ju*

The proband is a 22-year-old caucasian male of German ancestry in good health. No family history of disease was detected. The extended haplotypes are given in table 1.

3. RESULTS

3.1. *C4 immunoblot in controls*

With the exception of the proband, Rg:1-positive C4 allotypes were seen only in the acidic C4A region in 114 controls (frequency of *C4B*15* = 0.0088). No reactivity of anti-Rg:1 was found in either of our previously reported C4-deficient individuals [5,6].

3.2. *C4 immunofixation in pedigree Ju*

In the proband (II-1), an allotype which stains very faintly using polyclonal anti-C4 antibody can be seen in the C4B region (fig.1). This band is more easily detected in the mother (I-2) and is probably also present in II-2 as indicated by the width of the *C4B*2* precipitin band. The hemolytic activity of *C4B*15* was greater than *C4A*3* but clearly less than *C4B*1* or *C4B*2*. This rare allele segregates with the haplotype *HLA-A2 C-B50 BW6 DR7 DQ2 DR53/S07C2(1.12)* (table 1, fig.1).

3.3. *Anti-Rg:1 immunofixation in pedigree Ju*

After immunofixation with anti-Rg:, a strongly positive immunoprecipitin band can be seen in individuals I-2, II-1 and II-2, which is slightly slower than *C4B*2* (fig.2A). This band correlates with the faint

Table 1

Class I, II and III haplotypes in pedigree Ju

	HLA Haplotypes							Complotype
	HLA-A	*B*	*BW*	*C*	*DR*	*DQ*	*DRW*	*BF/C2/C4A/C4B*
Father								
A	1	8	6	7	3	2	52	SC01
B	28	18	6	–	2	1	–	SC42
Mother								
C[a]	2	50	6	–	7	2	53	S07C2(1,15)[b]
D	–	44	4	5	4	3	–	SC31

[a] haplotype carrying the *C4B* duplication
[b] parentheses indicate duplication of *C4B*

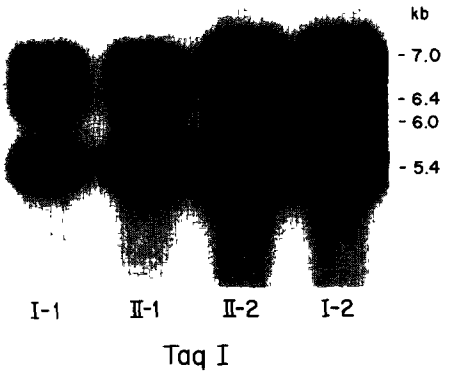

Fig.3. TaqI RFLP. ^{32}P-labelled pAT-A (5′ fragment) C4 probe was used to hybridize with DNA. In II-1, the 7.0- and 5.4-kb bands, representing fragments of the *C4A* and *C4B* genes, respectively, should be of equal intensity since the 6.4-kb fragment is a fragment of the single *C4B* gene on a haplotype with a *C4A* deletion. Note the relatively more intense 5.4-kb band in II-1, II-2 and I-2.

band seen by C4 immunofixation in these same individuals (not shown). By Western blotting, again using anti-Rg:1 monoclonal antibody, two immunoprecipitin bands are clearly seen in subjects II-1, II-2, and I-2, whereas a single precipitin band was present in the father (I-1) and the control (fig.2B).

3.4. *C4 genes in pedigree Ju*

In the DNA from the proband, TaqI restriction enzyme digestion yields of 7.0 kb, 6.4 kb and 5.4 kb fragment indicating the presence of only one *C4A* gene (7.0 kb fragment) (fig.3) since the 6.4-kb (on paternal haplotype A) fragment is characteristic of a haplotype with one short *C4B* gene with a *C4A* deletion. At least two C4B genes are present, as indicated by the detection of the 6.4-kb and 5.4-kb fragments. The relatively greater intensity of the 5.4-kb fragment (*C4B* fragment) as compared to the 7.0-kb fragment (*C4A* fragment) (ratio = 1.58 in proband II-1 or approximately 3:1) is consistent with the presence of three short C4B genes in an individual with *C4A*Q0 C4B*1/C4A*2 B*1,15* complotype. Since maternal haplotype C segregated with *C4* TaqI fragments of 7.0 and 5.4 kb, these results strongly suggest the presence of a *C4B* gene duplication on the maternal haplotype C.

4. DISCUSSION

A rare C4 allotype has been detected which is characterized by: (1) very faint reactivity with polyclonal anti-C4 antisera on agarose electrophoresis; (2) strong reactivity with MAB anti-Rg:1 antiserum; (3) electrophoretic mobility in agarose which is characteristic of a C4B allotype but with diminished hemolytic activity for a C4B allotype; and (4) segregation with an extended haplotype carrying a probable *C4B* gene duplication within the complotype (S07C2 [1,15]).

Diminished reactivity of a C4 allotype has been reported in a Lebanese pedigree with a S07C21 complotype [5] which is similar to the complotype in our pedigree. As was true in our case, the protein product was very difficult to detect by agarose electrophoresis but readily detected using monoclonal antibody to Rodgers. *C4B*15* was only slightly slower than the common *C4B*2* and may be part of the same complotype since reported in a Tunisian population by the same French group (*HLA-B50*, S07C[2,X]1) [8]. In addition, the analysis of TaqI RFLP of *C4* genes by the French group and in our laboratory has provided evidence of a *C4B* gene duplication on the haplotype which encodes the 'hypomorphic' C4 allotype.

The use of newer analytical tools to study the C4 system, such as the use of MAB anti-Rg:1, has consistently provided evidence of greater complexity but has also resulted in a better understanding of C4 [2]. For example, C4 allotypes with reversed Rodgers /Chido antigenicity (i.e. hybrid proteins) are now well described [9]. However, there are several questions raised by our detection of a Rg:1-positive C4B allotype which is 'hypomorphic', that is, difficult to detect by standard protein electrophoretic methods. Although we did not detect any such allotype in our two patients with total C4 deficiency, one can ask how common such hypomorphic allotype may be and what is the relationship of hypomorphic alleles with 'null' alleles or with total C4 deficiency? What is the biologic activity of such hypomorphic variants of C4, such as in virus neutralization or in immune complex clearance? Although *C4B*15* is hemolytically active, there is clearly less hemolytic activity than in *C4B*1* or *C4B*2*. Are other C4 allotypes which lack C4 epitopes (e.g. the *C4A* duplication encoding *C4A*2,3* is Rg:1,-2) [2] functionally different than other allotypes; are such allotypes important in the pathogenesis of diseases [10]? Further functional studies of purified C4 variants and characterization of the encoding genes are necessary to answer such questions.

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