C7 deficiency in an Irish family: a deletion defect which is predominant in the Irish

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SUMMARY

Human deficiencies of terminal complement components are known to be associated with increased susceptibility to *Neisseria meningitidis* infection. Polymorphic DNA marker studies in complement deficient investigations allow identification of haplotypes associated with the deficiency and enable the possible identification of heterozygote carriers of the defect. We report studies of an Irish family in which the index case had suffered recurrent meningococcal disease and was found to be deficient in the seventh component of complement (C7). The availability of all family members enabled us to determine the segregating haplotypes. The defects in the family segregated with two very closely related C6 and C7 DNA haplotypes, one of which is known to be associated with the large Irish C7 DNA deletion defect. The index case and two C7 deficient siblings were found to be homozygous for this defect, a deletion that spans approx. 6·8 kbp and encompasses exons 7 and 8. The deletion defect of exons 7 and 8 of C7 has been found in homozygous form in another C7 deficient Irish individual, and is present in heterozygous form in C7 deficient members of a third Irish family. Therefore, this deletion defect occurs in five of the six deficient chromosomes of these three unrelated Irish families, raising the interesting question of how prevalent this defect may be within the Irish community.

Keywords complement genetic defects DNA markers C7 deficiency *Neisseria meningitidis*

INTRODUCTION

The terminal complement system consists of components five to nine. Of these, components six to nine are related plasma proteins which differ in size and complexity [1]. Activation of the complement cascade results in the terminal complement components combining to form the C5b-9 complex. On cell membranes, this complex becomes the membrane attack complex (MAC) which is capable of forming trans-membrane channels through which ions migrate, leading to cell lysis and cell death [2].

Genetically determined human deficiencies of any of the terminal complement components are associated with increased susceptibility to *Neisseria meningitidis* infections [3–5]. These deficiencies are determined traditionally using haemolytic complement assays [6]. The genes for the sixth and seventh complement components (C6, C7) have been assigned to chromosome 5p13 [7]. These genes have been shown to be linked closely on this chromosome [8,9]. Both the C6 and C7 genes are polymorphic and are close homologues of the C8α, C8β and C9 genes [10]. Molecular bases of C6 and C7 deficiencies have been reported [11–13], as well as those of subtotal deficiencies of C6 and C7 [14].

A rapidly increasing number of polymorphic DNA markers for the C6 and C7 genes have been described [15–19]. DNA marker studies in complement deficient subjects allow the determination of genetic markers associated with deficiencies in individuals who lack protein markers [15,19–21]. Neutral DNA polymorphic markers arise from changes in the DNA sequence, usually by only one base pair, that do not result in the arrest or major alteration of protein synthesis. These are commonly detected by some form of restriction fragment length polymorphism (RFLP) assay. The use of DNA genetic markers can be very informative; neutral genetic markers can be used to detect linked genetic defects, as they directly assess the genotype rather than examine phenotypic features. Consequently, DNA marker studies are useful in detecting heterozygote carriers, as well as being indicators of the genetic heterogeneity of the defects [19,21]. Ten different polymorphic DNA markers have been reported for the C6 and C7 genetic region. These include three *Taq* I polymorphic sites [15,17,22], a C6 *Msp* I site [16], four C7 DNA polymorphic sites [17,18,20], a C6 A/B

Generation I

Marker	Parent I.1	Parent I.2
(i) C6 Dde I	B A	A B
(ii) C6 Msp I	– –	+ –
(iii) C6 Taq I	L L	L L
(iv) C7 Taq I (225)	L L	H L
(v) C7 Taq I (233)	L H	H H
(vi) C7 A/T	A T	A T
(vii) C7 M/N Rsa I	N M	M M
(viii) C7 12-27 A/C	C C	A C
(ix) C7 S367T	– +	– +
(x) C7 A/T Apo I	A A	T A

Generation II

	II.1	II.2	II.3	II.4	II.5	II.6	II.7	II.8
(i)	A B	A A	A B	A B	A A	A B	A B	B B
(ii)	– –	+ –	+ –	– –	+ –	+ –	– –	– –
(iii)	N/T	N/T	L L	N/T	L L	N/T	L L	L L
(iv)	N/T	N/T	H L	N/T	H L	N/T	L L	L L
(v)	H H	H H	H L	H H	H H	H L	H H	L H
(vi)	T T	A T	A A	T T	A T	A A	T T	A T
(vii)	M M	M M	M N	M M	M M	M N	M M	N M
(viii)	C C	A C	A C	C C	A C	A C	C C	C C
(ix)	+ +	– +	– –	+ +	– +	– –	+ +	– +
(x)	A A	T A	T A	A A	T A	T A	A A	A A

□ normal C7 haplotype   ■ C7 deficient haplotype   ◧ heterozygous C7 deficient   N/T not tested
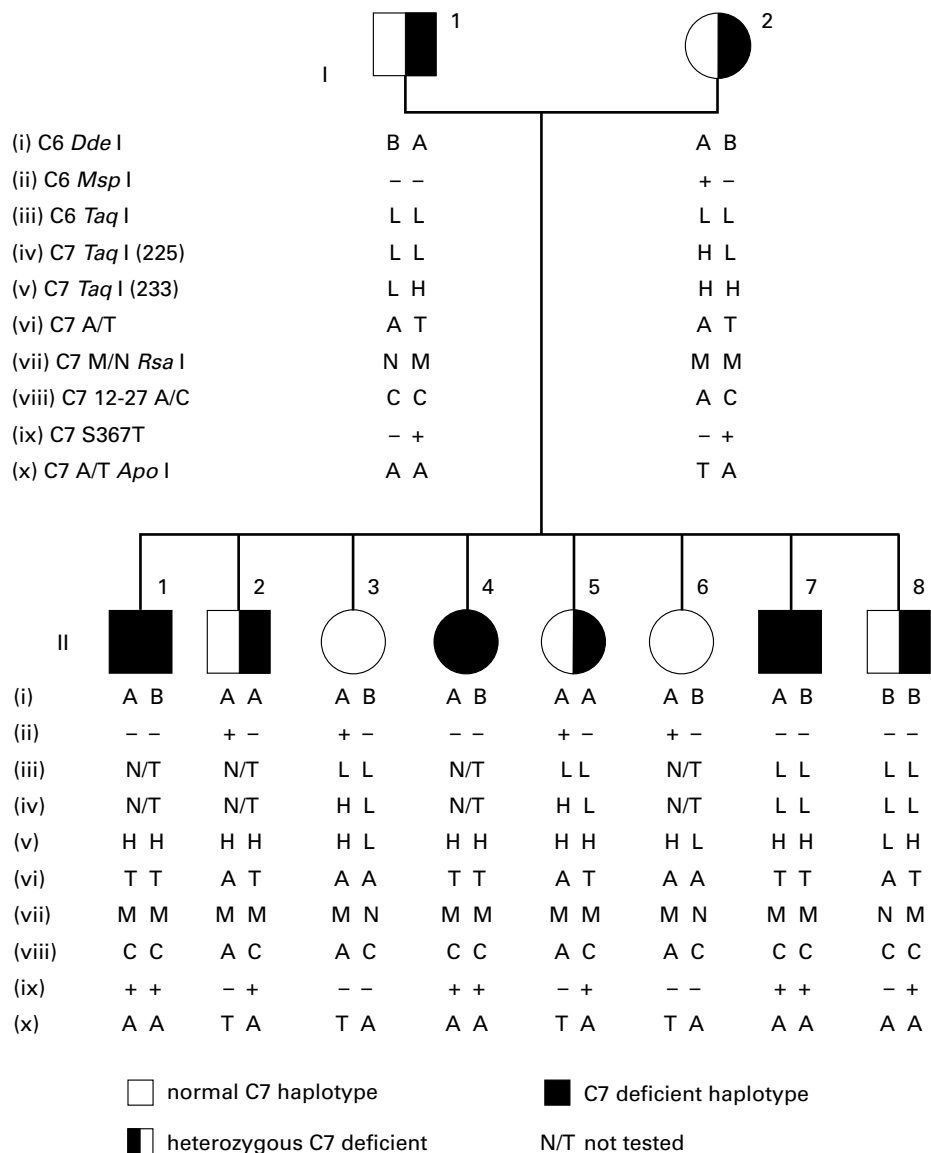
Fig. 1. Family A comprised parents and eight children. Subjects II.1, II.4 and II.7 are C7 deficient. The data on the markers are presented as vertical haplotypes.

polymorphism [23,24] and a C7 intron 1 Apo I site (Fernie et al. unpublished data). These 10 polymorphic DNA markers potentially generate 1024 different haplotype combinations.

The investigations reported here focused on the study of polymorphic DNA markers in an Irish C7 index case and his immediate family. The deficiency had been ascertained due to repeated meningococcal infections. The availability of all family members in the study enabled us to ascribe a particular DNA marker haplotype to the defective condition. The determination of C6/C7 DNA haplotypes of the index case in the marker study allowed us to proceed immediately to testing and confirming the occurrence of the C7 exon 7 and 8 deletion defect [13] in both his C7 genes. Confirmation that the C7 deletion occurs in a homozygous form in this family now means that this defect has been found in five of six deficient chromosomes in three unrelated families in the Republic of Ireland, posing the possibility that it may be a relatively common cause of C7 deficiency in this country.

SUBJECTS AND METHODS

Subjects
Family A was an Irish family that has not previously been reported and comprised both parents, four sons and four daughters (Fig. 1). The C7 deficient index case, subject II.7, had been ascertained because of meningococcal infections at the age of 16 years and again a year later.

Functional assays
Total functional complement activity and C7 functional activity in the serum of members of family A were tested by haemolytic assays and double diffusion haemolytic assays in 1% agarose gels [6,25].

DNA preparation
Genomic DNA was isolated for all 10 family members and controls from whole EDTA-treated blood by a modification of a method described previously [26].

Table 1. Oligonucleotide primers and products of exon polymerase chain reaction (PCR)

Location	5′ primer	Anneal temperature (°C)	Product size (bp)	3′ primer (5′→3′)
C6 intron 3	CCCAATTTTCCCAAGAAT	59	178	CCTTTTACCTTCTTGCCTGT
C6 exon 3	TTAGATCTGTCTTGCGTCCCAGTCAG	62	138	CGGGATCACAGCGAAATTTATTCTTGCAG
C7 exon 1	TCACTTTGTACCCCATAAATT	56	265	GTTGGAGGGATACACAGATTCA
C7 exon 2	ATTCTTTGTGTTCCCTTGCG	65	209	CCATTTTGGCCATGCCAA
C7 exon 6	TGCATTTGTGCCAATGAAGAGC	63	502	TGTGCCTGGCCAGTATAAAT
C7 exon 7	TTGGTTGATTGGAGATGAGAGC	66	401	CCCATTAAAATCCCAGAAATGCCT
Inside exon 7	TTGGTTGATTGGAGATGAGAGC	64	188	AGTCATACAGAGAGGGGAGGTGGG
Outside exon 7	CAATCTGGTCGTTAGGAGG	64	77	CCCATTAAAATCCCAGAAATGCCT
C7 exon 8	TCACTCTTGATTAGATGGCC	60	335	CCCCATGACACAAGTTTACC
Inside exon 8	TCACTCTTGATTAGATGGCC	59	173	AGCGTTTTCCAGTTCCTTGC
Outside exon 8	TCATGGATGCAAGGAACTGGAAAA	59	106	CCCCATGACACAAGTTTACC
C7 exon 9	GCTCTGCCTATTCATCCCTCCC	67	442	ATTGGGATACGGAAAATGCTAATT
C7 exon 11	TTGTTAGCAGGAAGCATAGC	60	372	AGTCCCTGGTAAAAATAGCC
C7 exon 13	GCTTGCCTGATGATTATGATTT	62	408	AGGCGGAGGGCTTATTC
ARMS/WT3	GCTTGCCTGATGATTATGATTT	59	56	AAAGTAAAAGATGGAAAAGC
ARMS/MU3	GCTTGCCTGATGATTATGATTT	59	56	AAAGTAAAAGATGGAAAAGA
C7 exon 15	AAGAGGCTTTTCTCCTAACG	60	230	GCTACACCTTCCATCCAACA
C7 exon 16	AACATCTGGGGGCACTAAGC	58	337	GTACTGTGACTTTAGCATTCTTAT
C7 exon 17	CCCAATTTCCTGGTCCTA	58	628	AAGGCCCAGAGAAGGTAA

Polymerase chain reaction

Exon-specific polymerase chain reaction (PCR) amplification [27] was performed on intron and exon 3 of C6, and on exons 1, 2, 6, 7, 8, 9, 11, 13, 15, 16 and 17 of the 18 C7 exons. Details of the primers used and the annealing temperatures are given in Table 1. Cycle conditions were 93°C for 1 min for denaturation, followed by 1 min at the specified annealing temperature. Extension was performed at 72°C for 1 min over 30 cycles in a Perkin-Elmer Cetus Thermal Cycler (Foster City, CA) or for 2 min at 72°C in an MJ Mini-Cycler (MJ Research Inc., Waterton, MA). dNTPs (50 μmol/l), 0·5 μmol/l of each primer and 0·5 U *Taq* DNA polymerase (Promega Corp., Madison, WI) were used with about 100 ng of template DNA in a final reaction volume of 50 μl. All amplifications were performed under mineral oil (Sigma-Aldrich Chemical Co., Poole, UK).

Amplification by PCR was confirmed by examination of 5 μl of PCR product on 2% agarose mini-gels in standard 1 × Tris-borate-EDTA buffer (0·09 M Tris-borate, 0·002 M EDTA) (TBE) with 0·5 μg/l ethidium bromide. The gels were subjected to electrophoresis at a constant current of 65 mA and subsequently visualized by ultra violet (UV) illumination.

Restriction digests

Genetic markers in PCR products were examined by restriction digests. The restriction enzymes *Dde* I, *Apo* I, *Rsa* I and *Bsr* GI were obtained from New England Biolabs (St Albans, UK). Digestion was carried out over 2–4 h at the specified incubation temperature using the supplied buffers according to the manufacturer's instructions.

Digests of C7 exon 9 with *Dde* I and C7 exon 13 with *Bsr* GI were examined following electrophoresis on 2% agarose/TBE mini-gels. Smaller DNA fragments were resolved by electrophoresis on either 10% w/v or 12% w/v (19:1 acrylamide:bis-acrylamide ratio) polyacrylamide gels in TBE, cast on Gel-Bond PAG Film (FMC Bioproducts, Rockland, ME). *Dde* I digested C6 exon 3 and *Apo* I digested C7 exon 1 PCR products were examined on 12% w/v polyacrylamide gels, whereas *Rsa* I digested C7 exon 13 and *Msp* I digested intron 3 of C6 products were resolved on 10% w/v polyacrylamide gels. Following electrophoresis all polyacrylamide gels were silver-stained using an adapted protocol [28] as outlined by Fernie *et al.* [24].

Southern blotting

Further restriction digests using the enzymes *Sac* I, *Hind* III and *Taq* I (New England Biolabs) were performed on approx. 10 μg of genomic DNA from selected members of family A and normal controls, adhering to the manufacturer's protocol, at a specified
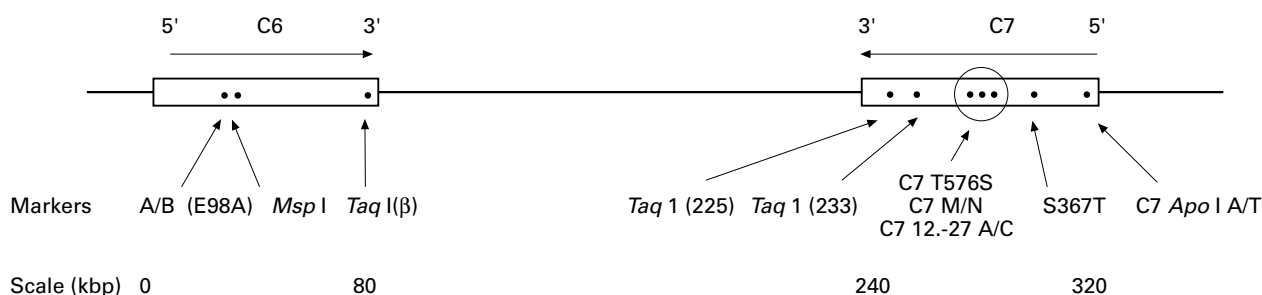

Fig. 2. Map of the C6 and C7 gene region of chromosome 5p13 showing the positions of the 10 polymorphic DNA markers used in this study.

Table 2. C7 deficient haplotypes of three unrelated C7 deficient Irish subjects

Marker/subject	Family A Subject II.7	Family 2 Subject II.2	Family 1 Subject II.1
C6 Dde I, exon 3	A B	B B	A B
C6 Msp I, intron 3	− −	− −	+ −
C6 Taq I, C6β/C6C, intron 16	L L	L L	H L
C7 Taq I, C7−225, intron 15	L L	L L	L L
C7 Taq I, C7−233, intron 13	H H	H H	H H
C7 A/T, exon 13	T T	T T	A T
C7 M/N, exon 13	M M	M M	M M
C7 A/C, intron 12	C C	C C	C C
C7 Dde I, exon 9	+ +	+ +	− +
C7 Apo I A/T, intron 1	A A	A A	T A

The deficient haplotypes of subject II.7 family A, and those reported previously by Fernie *et al.* [13], were assigned after family studies.

incubation temperature and using the buffers supplied. The samples were analysed on 0·8% agarose submarine gels in 0·04 M Tris-acetate/0·001 M EDTA buffer and blotted overnight [29] onto Hybond N filters (Amersham International, Aylesbury, UK). The filters were UV cross-linked in a Stratalinker (Stratagene, Cambridge, UK) before hybridization to ^{32}P-labelled C6 or C7 cDNA probes. The probes were radiolabelled using the Ready-to-Go DNA Labelling Kit (Pharmacia Biotech, St Albans, UK) according to the manufacturer's instructions. Hybridization was conducted under standard conditions [30]. Washing stringency was 0·4× sodium chloride sodium citrate/0·1% SDS at 65°C and the filters were autoradiographed under enhancing conditions [31].

Investigation of C6/C7 marker haplotypes
DNA marker studies were performed to determine the C6/C7 haplotypes of the family in order to establish if they were likely to be homozygous or heterozygous for the molecular defect. The arrangement of the genes and the positions of the polymorphic markers on the chromosome are shown in Fig. 2.

The C6 A/B marker was determined by digestion of C6 exon 3 product with *Dde* I, as the responsible polymorphic site is at position 56 of the exon [23,24]. The PCR product of intron 3 of C6 was digested with *Msp* I [16]. The markers C6β *Taq* I (adjacent to C6 exon 17); C7-225 *Taq* I (C7 intron 15); and C7-233 *Taq* I (C7 intron 13) (where β, -225 and -233 indicate the C6 or C7 cDNA probe used) were determined via Southern blotting [29] as described earlier [15,17,22]. The C to G polymorphism at position 367 of C7 exon 9 leads to a threonine/serine substitution. The polymorphism was investigated using *Dde* I digested PCR product (C7 S367T) [17]. The C7 A/T polymorphic marker was determined by digestion of amplified exon 1 and part of intron 1 of C7 with *Apo* I, as within the amplicon there is a T to A polymorphism at position 289 of intron 1 (Fernie *et al.*, unpublished data).

The PCR product of C7 exon 13 was digested with both *Rsa* I and *Bsr* GI. The base change responsible for the C7 M/N protein polymorphism [32], at position 363 of the exon, was detected by *Rsa* I digestion [33]. The adjacent A→T point substitution at position 396, resulting in the C7 T576S polymorphism, was detected by *Bsr* GI digestion of the same PCR product [19]. The final marker was a C→A point substitution at position 327 in intron 12 of C7 (C7 12.-27) only 36 bp 5′ of C7 M/N. This marker was detected by the amplification refractory mutation system (ARMS) using allele-specific primers [18,34]. The nomenclature

for the alleles investigated in the DNA marker study are those used previously [19]. These 10 bi-allelic markers define 1024 possible haplotypes in the C6/C7 gene region.

RESULTS

Functional assays
Total complement functional assays, C7 functional assays and double diffusion haemolytic assays indicated a lack of functional C7 in subjects II.1, II.4 and II.7 of the family (Fig. 1), whereas all other family members were found to be complement sufficient.

Marker studies
A total of 10 polymorphic DNA markers in the C6 and C7 gene regions were investigated (Fig. 2). The inclusion of all 10 family members in the marker studies enabled us to ascribe a particular DNA marker haplotype to the defective condition and to construct a family tree for family A (Fig. 1). It was found that of the eight siblings; two sons and one daughter are C7 deficient (subjects II.1, II.4 and II.7), two sons and one daughter are heterozygous carriers of the deficiency (subjects II.2, II.5 and II.8), whereas the other two daughters are homozygous C7 sufficient (subjects II.3 and II.6). The proband (subject II.7) and his deficient brother and sister (subjects II.1 and II.4) are homozygous for all C7 markers and all but one of the C6 markers (C6 exon 3, *Dde* I).

PCR amplifications
The presence of intron and exon 3 of C6, and exons 1, 2, 6, 9, 11, 13, 15, 16 and 17 of C7 was confirmed in all family members by examination of PCR products following electrophoresis. Attempts at PCR amplification of exon 7 and exon 8 of C7 failed in all three C7 deficient siblings, but not in any complement sufficient family members. In order to confirm that the PCR failures of exons 7 and 8 were not due to localized absence or modification of the intron sequences of the template, further PCR reactions were carried out between primers within the exons and those in the flanking introns. These also failed, indicating the probable deletion of both exon 7 and exon 8 in deficient members of family A.

Similar PCR failures of exons 7 and 8 of C7 have been reported for another C7 deficient Irish individual [13], who is homozygous for all of the C6 and C7 markers. The 'deficient' haplotype of this subject (Table 2, subject II.2, family 2) is identical to one of those presented here for the index case of family A (Table 2). Therefore,
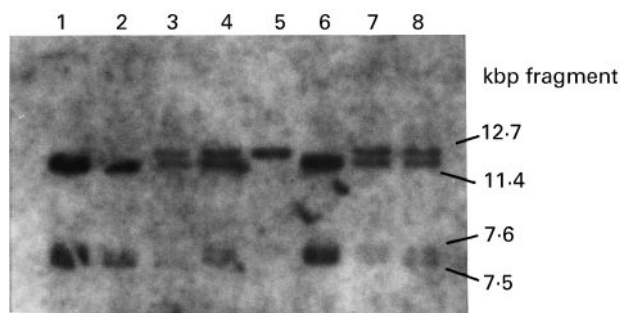
Fig. 3. *Sac* I Southern blot showing the presence of a 12·7-kbp fragment in the proband (lane 5) and a 11·4-kbp fragment in normal individuals (lanes 1, 2, 6). The parents (lanes 3, 4) and heterozygous siblings II.5 and II.8 (lanes 7, 8) reveal both the 11·4-kbp and 12·7-kbp fragments. The 7·5-kbp and 7·2-kbp fragments are revealed due to the presence of other *Sac* I restriction sites along the chromosome [13].

to establish if the molecular basis of the defect in these two families is also identical, we proceeded to analyse for the C7 exon 7 and 8 deletion defect.

Southern blotting—verification of exon deletions
Southern blots using *Hind* III, *Sac* I or *Taq* I restriction digests were used to investigate the extent of the deletion of exons 7 and 8 in the proband. *Hind* III blots are informative, as exons 8 and 9 are contained in individual *Hind* III fragments, and in the normal chromosome a *Hind* III fragment containing exons 6 and 7 is about 14 kbp [13]. The size of this fragment is not significantly different if exons 7 and 8 are deleted, as the fragment then presumably terminates at a *Hind* III site just 5′ of exon 9 [13]. Probing of a *Hind* III blot with an exon 7 probe revealed no band in the proband, but hybridized to the normal 14-kbp fragment in complement sufficient family members (data not shown). This confirms a deletion of exon 7 in subject II.7. A similar approach using an exon 8 probe is not practical, as the probe contains repetitive sequences and would hybridize to a smear.

A *Taq* I blot probed with C7-401 3′ cDNA, which contains exons 4–12, reveals a number of bands including a 1·9-kbp fragment, which is seen in the index case, his parents and heterozygous siblings, but not in subject II.3, a normal sister. Another 2·65-kbp fragment containing the 5′ half of exon 7 is absent in subject II.7, but present in all normal and heterozygous family members examined (data not shown).

The *Sac* I digest was particularly informative. Subject II.7 of family A produces a band of 12·7 kbp but not the 11·4-kbp band which is revealed by normal individuals (Fig. 3). However, his parents and heterozygote siblings reveal both the 12·7-kbp and 11·4-kbp band fragments (Fig. 3). As reasoned by Fernie *et al.* [13], a detailed examination of these band fragments and the positions of the restriction sites of *Sac* I on the exon [13] indicated that the deletion of exons 7 and 8 of C7 spans approx. 6·8 kbp.

DISCUSSION

It is possible to provide a complete explanation for the C7 deficiency in family A from the presented data. The apparent deletion of exon 7 and exon 8 of C7, highlighted initially by PCR failure, was subsequently confirmed in two ways. First, PCR reactions between primers within the exons and those in the

flanking introns also failed, and second, probing of a *Hind* III blot with an exon 7 probe revealed no band in affected family members. This is satisfactory confirmation of the absence of a gene segment in these subjects. These results, in addition to those obtained in the *Sac* I and *Taq* I digested blots, are similar to those previously published for an unrelated C7 deficient Irish individual (subject II.2, family 2) [13]. Fernie *et al.* [13] conclude that this deletion of exons 7 and 8 is approx. 6·8 kbp, where the 5′ boundary is 800–1400 bp 5′ of exon 7 and the 3′ boundary is 1200–1800 bp 5′ of exon 9. Such a deletion is sufficiently large to be responsible for the homozygous C7 deficiency in affected members of family A and is the largest defect reported to date in a terminal complement component deficient gene.

The deficient haplotypes of family A are shown in Table 2. Also shown for comparison are the deficient haplotypes of two other unrelated C7 deficient Irish individuals who had suffered recurrent meningococcal infections. These subjects have been reported previously by Fernie *et al.* [13], as the index cases in families 1 and 2 and their deficient haplotypes had been determined and assigned by similar marker studies [19]. However, the deficient haplotypes of these individuals have now been extended to include the C7 intron 1 *Apo* I polymorphic site (Table 2).

The C7 deficient members of family A are homozygous for all C7 markers and all but one of the C6 markers (C6 exon 3, *Dde* I). The deficient haplotype B-L; LLHTMC+A, found in the mother (subject I.2) and four siblings (subjects II.1, II.4, II.7 and II.8) is identical to those reported for subject II.2, family 2 [13]. This subject is homozygous for all of the C6 and C7 markers, including the C7 intron 1 *Apo* I polymorphic marker not investigated in the previous study. This C7 deficient haplotype is present also in heterozygous form in subject II.1 of family 1 (Table 2) [13].

The other very closely related deficient haplotype of family A, A-L; LLHTMC+A, is present in the father (subject I.1) and five of his offspring (subjects II.1, II.2, II.4, II.5 and II.7). This deficient haplotype differs from the other deficient haplotype of the family and those previously described (subject II.2, family 2 and subject II.1, family 1) [13] in only one polymorphic marker site, C6 exon 3 *Dde* I A/B. However, given the position of this marker site on the chromosome (Fig. 2), the possibility of a recombination event occurring between this and the next marker site is entirely feasible. Therefore, these two very closely related haplotypes occur in five of the six deficient chromosomes in three unrelated Irish families, and in each instance are associated with the exon 7 and 8 deletion defect. This raises the interesting question of how prevalent the deletion defect may be within the Irish community. The occurrence of the same C7 defect on two different C6/C7 haplotypes does suggest that this is an ancient defect, reaching far back into Irish history [35]. However, we have insufficient data to estimate how ancient the defect is or how long it has been segregating in the Irish population.

C7 deficiency, like C8 deficiency, appears to bear no selective advantage or disadvantage in heterozygotes [36]. Other reported investigations of patients with C7 deficiency reveal a wide variety of C7 haplotypes in sporadic cases of widespread geographical origin. Heterogeneous molecular defects leading to C7 deficiency in Japan have been described, although the two patients were homozygous for their particular defect [12]. However, many of the defects appear homogeneous for individuals living in defined geographical areas [19], and we have observed several predominantly homogeneous C6 and C7 defects among the Moroccan Israelis [19], and in the Western Cape, South Africa [37].

In family A, subjects II.1 and II.4, both C7 deficient, have not to date developed meningococcal infection, highlighting that deficiency is not incompatible with apparently normal health [5]. Diagnosis of underlying deficiency in patients presenting with meningococcal infections does allow appropriate precautions to be taken to minimize the risk of further infections [25]. DNA polymorphisms can be detected irrespective of whether the sequence change affects the phenotype, and prior knowledge of the molecular defect responsible for the deficiency is not required. Thus, DNA marker studies in investigations of affected families enables identification of both heterozygous and homozygous individuals. Identification of heterozygous carriers within affected families allows for the provision of informed genetic counselling and medical advice. Investigations of complement function and identification of possible complement deficiencies are important as part of the immunological assessment of subjects showing increased susceptibility to serious infections, including infections by *N. meningitidis*.

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