THE GENETIC POLYMORPHISM OF THE FOURTH COMPONENT OF HUMAN COMPLEMENT: METHODOLOGICAL ASPECTS AND A PRESENTATION OF LINKAGE AND ASSOCIATION DATA RELEVANT TO ITS LOCALIZATION IN THE HLA REGION*

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Component of human complement (C4)[1] has been isolated and studied in detail (1). It has a mol wt of about 204,000 daltons, and is composed of three distinct polypeptide chains joined by disulfide bonds and noncovalent forces. The α-, β-, and γ-chains have mol wt of approximately 93,000, 78,000, and 33,000 daltons, respectively.

Heterogeneity of C4 was first reported by Rosenfeld et al. (2), and in 1971, further observations were published by Bach et al. (3). By using crossed immunoelectrophoresis in agarose they could discern varying C4 patterns in different individuals. These patterns were identical in the same person studied on different occasions. Although the existence of a genetically determined polymorphism was suspected, it was concluded that the patterns obtained in family studies were not compatible with simple codominant inheritance of alleles at an autosomal locus and were not readily explainable by other genetic mechanisms.

By immunofixation electrophoresis in agarose gel we have recently demonstrated a structural polymorphism of human C4 (4). Preliminary studies showed that the polymorphism was governed by at least three codominant alleles at a locus in the major histocompatibility complex of chromosome 6. It is the purpose of this report to present more comprehensive data on the nature of this polymorphism as revealed by immunofixation electrophoresis and crossed immunoelectrophoresis in agarose gel, and by a specific hemolytic technique for protein visualization after electrophoresis in agarose. Linkage and association data are also presented.

Materials and Methods

The population material consists of unrelated adults from the family material. The family material comprises 34 matings with 154 children ascertained for dermatological disease, renal

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[1] *Abbreviations used in this paper*: C4, component of human complement; F, fast anodal band; FS, broad band; S, slow cathodal band.

disease, or for large size only. The complete material has been typed in 25–30 different marker systems and evidence of nonpaternity has not been found in the families presented here. Blood samples were either collected without additives or as heparin blood. Serum or plasma was stored at −75°C until tested.

Electrophoretic separation of plasma or serum proteins was performed essentially as earlier described (5). 3 μl of a 1:3 dilution of plasma or serum in saline were applied to each gel slot. There is normally a rather large variation in the C4 content of the blood of different individuals. It was therefore occasionally necessary to use other dilution factors. Concentrations of gel buffer used for electrophoresis were: barbitone-Na, 0.0230 M; barbitone, 0.0037 M; calciumlactate, 0.0009 M, pH 8.6. Concentrations for vessel buffer were: barbitone-Na, 0.0610 M; barbitone, 0.0106 M; calciumlactate, 0.0018 M, pH 8.6. Voltage used was 20 V/cm. Electrophoresis time was 1½ h. Cooling of the gel was achieved through use of a metal plate perfused with tap water. For prolonged electrophoretic runs (suggested by G. Mauff, Cologne, Germany) it was essential to use large buffer vessels, each about 1,000 ml. The prolonged 4-h electrophoresis experiments were mostly performed with a discontinuous Tris/citric acid/boric acid buffer system. Gel buffer concentration was 0.025 M, with a pH of 8.65. Vessel buffer concentration was 0.05 M, while its pH was 8.65.

Immunofixation was performed by layering undiluted antibody on the gel which was then incubated at 37°C in a moistening chamber for 1 h. The gel was washed for 48–72 h in saline before it was dried and stained in Coomassie Brilliant Blue.

Crossed immunoelectrophoresis was performed as follows: the proteins were separated by high voltage electrophoresis in agarose gel for 1–3 h as described above. A 1-cm wide strip of agarose containing the separated C4 proteins was removed and placed in a preformed slot in an anti-C4-containing gel. The second electrophoretic step was then performed for 1 hr under conditions identical to those of the initial separation.

The C4 bands were also visualized after electrophoresis by a functional in-gel hemolytic assay. Electrophoresis conditions were as described above. Liquid 0.8% agarose (type HSA, Litex, Glostrup, Denmark) with 0.75% sensitized sheep erythrocytes in complement fixation diluent containing 0.7–1% ammonia-treated guinea pig serum (R4) was layered on the separating gel in the C4 region. Bands of C4 hemolytic activity then appeared after ≅ 30 min.

Lastly, C4 patterns were visualized by the functional assay after isoelectric focusing in 5% polyacrylamide gel slabs. Ampholines (pH 5–7) were incorporated in the gel at a final concentration of 5%.

Specific anti-C4 was produced by immunization of rabbits with isolated C4. C4 was purified by ion exchange chromatography, ammonium-sulfate precipitation, and preparative electrophoresis on polyacrylamide gel. This antibody solution gave one precipitation arc upon immunoelectrophoresis and crossed immunoelectrophoresis against whole human serum. It showed, moreover, reactions of complete identity with two different commercially obtained anti-C4 preparations and against a third anti-C4 obtained from Dr. C. Alper, Boston, Mass.

Bf typing was performed by immunofixation after high voltage agarose gel electrophoresis (6). Glyoxalase I types were revealed by starch gel electrophoresis of hemolysates followed by specific enzyme-staining procedures. HLA typing for 9 antigens of the HLA-A series, 18 antigens of the HLA-B series, and 4 of the HLA-C series (part of the material only) was performed with techniques described previously (7). Typing for HLA-D determinants was performed on a minor part of the family material by mixed lymphocyte culture with homozygous typing cells (8).

Linkage data are presented assuming no double recombinants and no sibship with more than one recombinant. Tests for association were performed by the χ^2 or by Fisher's exact method. The calculated P values have not been corrected for the number of association tests made.

Results

Using the described immunofixation technique, we found that one of three C4 patterns was initially observed in the fresh plasma or serum from most individuals. These were: one slow, cathodal band only (S); one fast, anodal band only (F); or a broad band (FS). Additional patterns which were occasionally seen included a band of intermediate mobility in combination with either the S or the F band. Different bleedings from the same individuals always showed identical patterns.

A number of samples could not be typed by the short-run electrophoretic technique. We therefore introduced two technical modifications in the study of this obvious heterogeneity of human C4: crossed immunoelectrophoresis and extended electrophoresis as described above. To visualize C4 bands after extended electrophoresis it became necessary to employ the sensitive functional assay. All samples not typable with the short-run technique and the samples where the slightest doubts about the types existed were examined by these methods. A new C4 pattern, F_1, was then demonstrated, and it could be detected in combination with both the S and the F bands. Crossed immunoelectrophoresis shows F_1 as a broad, notched and slightly anodally situated peak, whereas extended electrophoresis shows it as a broad 5–6 band pattern occupying both the S and the F regions.

The modified techniques also gave new information regarding the previously described C4 types. Upon crossed immunoelectrophoresis, the S and F patterns appear as narrow symmetrical peaks located in the cathodal, and anodal regions respectively. The FS pattern showed, as expected, a broad symmetrical peak. After extended electrophoresis in the barbitone or Tris/borate buffer systems, the functional assay revealed multi-band C4 patterns. C4S showed at least three cathodally situaded bands while C4F were represented by at least three anodally situated bands. The FS pattern showed at least six bands.

The patterns obtained after isoelectric focusing in polyacrylamide gel slabs and functional visualization in the gel revealed a number of C4 bands, but this technique has not yet allowed us to perform C4 typing.

C4 patterns are best detectable in heparin or EDTA plasma. Storage for years at −75°C or repeated freezing and thawing did not preclude the use of plasma samples for C4 typing. Completely fresh serum or serum stored at −75°C may be used, but some degree of conversion with the formation of anodally migrating conversion products may be seen. This phenomenon may disturb the interpretation of the patterns and is the main reason that a minor part of our family material could not be C4 typed.

When heparin plasma is used, one should be aware of the appearance of a cathodally situated band taking protein stain after electrophoresis. It is not functionally or immunologically identical with C4 and can be seen also after electrophoresis without subsequent immunofixation, for instance upon C3 typing. Its nature is unknown to us, but it does not lead to typing problems.

We have investigated 34 matings with 154 children regarding C4 patterns. An additional seven matings showed C4 patterns that were not readily characterized, and these have not been included in the present material. As stated above, the chief reason why some samples could not be typed was the apparent degradation of C4 in vitro.

Typing results are shown in Table I. On the basis of the results we have postulated that the described heterogeneity of human C4 is governed by at least three common and one or more less-common alleles at one autosomal locus. The alleles demonstrated so far have been named *C4S*, *C4F*, *C4F*$_1$ and *C4M*. Figs. 1 and 2 show the inheritance of the common S and F bands in two families. The appearance of the three frequent phenotypes (S, FS, and F) after short run electrophoresis and immunofixation is illustrated on these figures.

TABLE I
C4 Types in 34 Norwegian Families with 154 Children

Matings*		Children								
Type	No.	No.	C4 types							
			F	F_1F	F_1	FS	F_1S	S	FM	MS
F × F	2	8	8							
F × F_1F	2	8	6	2						
F × F_1	1	5		5						
F × FS	7	40	16			24				
F × F_1S	2	10		7		3				
F × S	1	4				4				
F_1F × FS	5	13	4	4		2	3			
F_1F × F_1S	2	6		1	1	1	3			
F_1F × S	1	3				2	1			
F_1 × S	1	6					6			
FS × FS	2	5	1			4				
FS × F_1S	2	8		1		3	3	1		
FS × FM	3	25	7			9			4	5
F_1S × F_1S	1	3					2	1		
F_1S × S	2	10					6	4		
	34	154	42	20	1	52	24	6	4	5

* Some matings are from the same pedigrees.

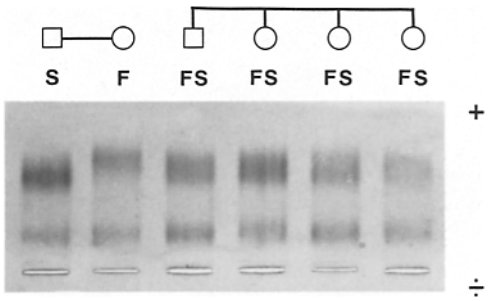

FIG. 1. C4 patterns revealed by immunofixation electrophoresis in an S × F mating with FS children. The single, narrow band seen close to the well is a contaminant of non-C4 nature.

Fig. 3 shows the inheritance of the F_1 band in a family. The types in this family were not easily revealed by immunofixation alone as the F_1 allele product in the heterozygous children became broad and indistinct. Upon crossed immunoelectrophoresis, however, it could clearly be seen that F_1 was present: the C4 peaks were broad and asymmetrical with anodal extensions. Fig. 4 shows the common C4 types as revealed by extended electrophoresis followed by functional visualization of the bands. In Fig. 5 the inheritance of the F_1 allele in a pedigree as revealed by extended electrophoresis and functional visualization is demonstrated. The typing results and gene frequencies in 51 unrelated adults from the family material appear from Table II. The number is small and includes only individuals who have been typed by all of the described

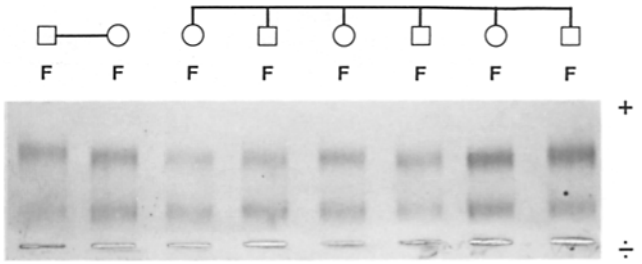

FIG. 2. C4 patterns revealed by immunofixation electrophoresis in an F × F mating with F children. The single, narrow band seen close to the well is a contaminant of non-C4 nature.

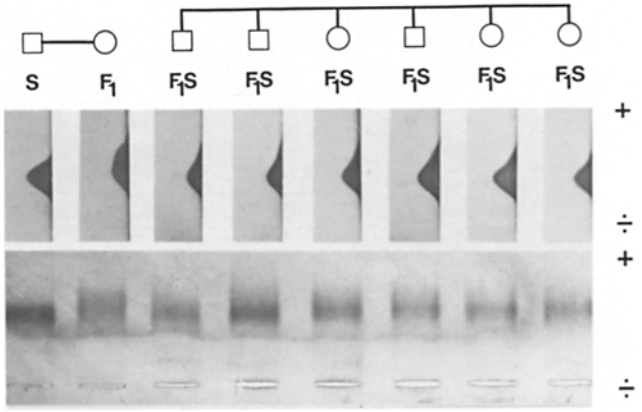

FIG. 3. C4 patterns as revealed by immunofixation electrophoresis (below) and crossed immunoelectrophoresis (above) in an S × F_1 mating with F_1S children. The F_1 allele product is revealed in the children as an anodal extension of the C4 peak on crossed immunoelectrophoresis.

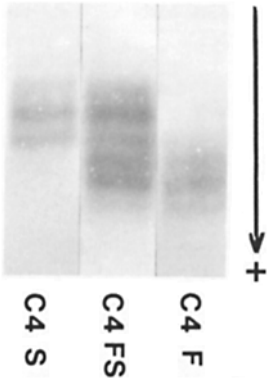

FIG. 4. C4 patterns as revealed by the extended electrophoresis system followed by functional detection of C4 protein bands. In the homozygous types three different bands can readily be seen. On the original plate at least another two bands may be detected.

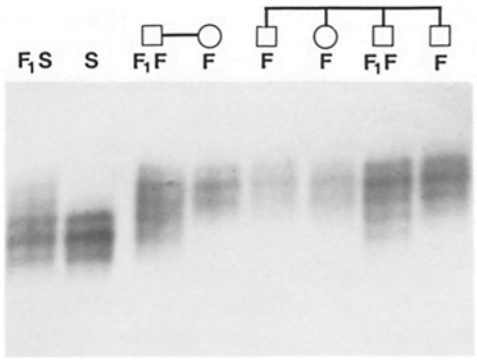

FIG. 5. Segregation of C4 in a family as revealed by prolonged electrophoresis and hemolytic in-gel assay. An F × F_1F mating has both F and F_1F children; the C4S and F_1S types are reference plasma samples.

TABLE II
Distribution of C4 Phenotypes in 51 Unrelated Parents

	C4 types									
	F	F_1F	F_1	FS	F_1S	S	FM	F_1M	MS	M
Observed	10	7	2	18	9	3	2	0	0	0
Expected*	10.8	9.4	2.0	15.0	6.5	5.2	0.9	0.4	0.7	0.0

C4 allele frequencies
C4F: 0.46, *C4F*$_1$: 0.20, *C4S*: 0.32, *C4M*: 0.02

* Assuming Hardy-Weinberg distribution.

TABLE III
Linkage Relations between C4 and HLA Region Markers

Nonrecombinants*:Recombinants					
HLA–				Bf	GLO
A	C	B	D		
155:3	48:0	165:0	7:0	53:0	35:2

* Some two-generation families are included. They have been scored assuming no double recombinants and no sibship with more than one recombinant.

techniques. The typing results have been verified through family segregation. Comparison with expected Hardy-Weinberg distribution is given in the table. There was a slight discrepancy between the typing results after short-run electrophoresis and those obtained after crossed immunoelectrophoresis and extended electrophoresis. A few individuals with the F_1S, F_1, and F_1F types had been typed S, FS, and FS respectively, and a few others who had previously been considered untypeable were assigned C4 types.

The linkage data are given in Table III. The data are now quite extensive with regard to the relationship between the *C4* locus and *HLA-A* and *B*, and strongly suggest that *C4* is situated very close to *HLA-B*. In all three families

with known breaks between *HLA-A* and *-B*, *C4* follows *-B*. Information regarding the relationship between *C4*, *Bf*, and *GLO* also support this conclusion. We have not yet been able to study families with known breaks between *HLA-B* and *-D* informative in regard to *C4*.

The association data are particularly interesting: all 12 *B8* haplotypes are inherited together with *C4S* ($P < 0.0005$); of 20 *B12* haplotypes, 16 are found together with *C4F* ($P < 0.005$); and of 8 *B7*, seven are found with $C4F_1$ ($P < 0.0005$).

20 out of 25 *BfF* alleles go with *C4F*, whereas 18 out of 20 $C4F_1$ alleles go with *BfS*, and 28 out of 32 *C4S* alleles go with *BfS*. The distribution of *Bf* alleles among the different *C4* alleles thus deviates from expected values ($P < 0.001$).

The association between products of *HLA-A* and *C4* is loose and of a magnitude which could only be expected from the assumption that *C4* is closely linked to *HLA-B*. The data on HLA-C and D determinants are scarce and allow no conclusions regarding association.

The distribution of *GLO* and *C4* alleles does not deviate from expected values ($P < 0.20$) in accordance with the *HLA-B-GLO* distance (3% in our data).

Discussion

Immunochemical studies of plasma from unrelated individuals have shown different C4 patterns. The patterns are reproducible in various bleedings from the same individuals. Family studies have shown that the protein bands comprising the C4 patterns in the parents are distributed among the children as if determined by alleles at one autosomal locus. We therefore believe to have demonstrated a genetically determined, structural polymorphism of human C4.

This polymorphism may be regarded as a system with three common alleles. Thus the variability of the system is large. More than half the population is expected to be heterozygous at this structural *C4* locus. This is an obvious advantage for a system which is to be used in linkage and chromosomal assignment work.

In a short previous report (4) we described only two common alleles. We have now been able to demonstrate a new, frequent allele, $C4F_1$. This allele was probably missed in some individuals of the $C4F_1S$ constitution, as the narrow and dense S pattern dominated completely. This may have led to the higher *C4S* frequency reported in the preliminary study. The multi-band patterns obtained with the extended electrophoretic techniques do not change our concepts of C4 genetics.

The typing problems which may be encountered in work on the C4 system are usually of little consequence in linkage work. Most families show simple inheritance of bands in the F and/or S region. It is at present more difficult to apply a system of this kind to population genetic studies, and the system should not, with the presently available techniques, be used in forensic genetics. The typing techniques are, however, rapidly improving. When extended electrophoresis with the functional detection system, which we now strongly recommend, has been in routine use for some time, we may be able to alter this conclusion.

Structural polymorphism is at present known to exist also in complement components C2 (9, 10), C3 (11), C6 (12), C7 (9), C8 (C. Alper, personal communication) and in factor B of the alternative pathway of complement activation (13). These genetic polymorphisms have made it possible to perform linkage studies in pedigrees. By investigating the segregation of these polymorphic traits and of genetic defects in complement component levels, it has been possible to assign the loci of C2 and factor B to chromosome 6 in man (10, 14, 15). C3 (16), C6 (17, 18), and C7 (M. Hobart, personal communication) are probably not coded by loci on chromosome 6, and they are at least not linked to the major histocompatibility complex.

Our data show that the *C4* locus is situated very close to the *HLA-B* locus on chromosome 6. In the family material no recombinants have been found between the two loci. In three families with known cross-overs between *HLA-A* and *HLA-B*, *C4* follows *B* in each instance. The data also show strong association between C4 and HLA-B types. These results strongly suggest that C4 is situated on the *B* side of *HLA-D*. We do not know on which side of *HLA-B* *C4* is localized. This also places the *C4* locus very close to the *Bf* locus (locus for factor B), a suggestion confirmed by the association between C4 and Bf types shown in this report. Most data now locate the *Bf* locus close to *HLA-B*, to the right of *HLA-D* (19–22).

The two families recently reported with genetic defects in blood levels of C4 may well have silent or null alleles at the presently described structural *C4* locus (23, 24).

The exact localization of *C2* is at present not definitively established, but it is situated in the major histocompatibility complex and may well be extremely close to *C4* and *Bf*. The three proteins are closely related in function, and their loci may have evolved from a common ancestor by gene duplications and point mutations. Studies in mice (25, 26), guinea-pig (27), and rhesus monkey (28) indicate that this chromosomal segment may have changed very little during a long period of mammalian evolution.

The close linkage of loci determining important factors, both of the recognition and the effector parts of the immune system, may have implications for the deeper understanding of immune system function.

Summary

The C4 polymorphism in man has been studied by immunofixation electrophoresis, crossed immunoelectrophoresis, and functional detection after agarose gel electrophoresis. It has so far not been possible to reveal this polymorphism by isoelectric focusing and functional detection of C4 bands.

Three common alleles and one less frequently occuring allele have been identified. In a small population sample studied by all the different techniques and verified by family segregation, the following gene frequencies have been found: *C4F*: 0.46, *C4S*: 0.32, $C4F_1$: 0.20, and *C4M*: 0.02.

By linkage and association studies in a family material it has been shown that a structural *C4* locus is situated in the *HLA* region of chromosome 6 very close to the *HLA-B* and *Bf* loci.

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