ALLOTYPES OF THE FOURTH COMPONENT OF COMPLEMENT IN HEALTHY AND DISEASE FAMILIES

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ALLOTYPES OF THE FOURTH COMPONENT OF COMPLEMENT IN HEALTHY AND DISEASE FAMILIES

by

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A Thesis presented in partial fulfillment
of the requirements for the degree of
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ABSTRACT

1857 serum samples from healthy individuals, patients, and their families were characterized with respect to C4 polymorphic variants, the relationship between genotype and C4 concentration, associations between C4 haplotypes and other MHC variants, and the inheritance of C4 and other MHC allotypes in multiple sclerosis (MS) and insulin-dependent diabetes (IDDM) families.

Patterns obtained were consistent with (i) two gene products, C4A (Rg+) and C4B (Ch+), (ii) two loci, A and B, per chromosome, (iii) at least five alleles at each locus, A3 and B1 being the most common, and (iv) moderately frequent null alleles, AQO and BQO. Some individuals with the rare patterns A3A2 and B2B1 appeared to have two A or two B genes per chromosome. For two rare products, B3 and B4, the appropriate Chido antigen could not always be demonstrated.

Null alleles could not be excluded from many A3B1 phenotypes. Measurement of relative amounts of C4A and C4B products per individual and of total serum C4 is of limited value in predicting the number of genes an individual possesses. Factors other than MHC-linked genes are likely to be important in determining serum C4 levels.

Frequencies of C4 haplotypes and of C4*A and C4*B genes were estimated from 1048 founder haplotypes. Five A-B

combinations had frequencies > 2% and were non-randomly associated.

Many MHC alleles, C4-C2-BF complotypes, and extended MHC supratypes showed high positive linkage disequilibria. The associations suggest that the C4 loci are between HLA-B and HLA-DR, closer to HLA-DR. The frequent clustering of rare C4, BF, and C2 alleles in the same supratype indicates that some may be hypermutable.

Supratypes of patients were compared with non-disease supratypes from the same families. HLA-B7 C4*A3B1 BF*S C2*1 HLA-DR2 was increased in MS-patient supratypes. This is likely to reflect strong associations among these alleles and between MS and HLA-DR2 suggesting that an MS susceptibility gene is very closely linked to HLA-DR. C4*AQOB1 and C4*B3 occurred more frequently in IDDM supratypes. These associations may also be secondary to strong associations reported for IDDM and HLA-DR or, because other rare complement variants are associated with IDDM, may indicate a direct role for particular complement variants in this disease.

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CHAPTER I INTRODUCTION

A. The complement system

Activated components of the complement system constitute an important effector arm of the immune system, carrying out such biological activities as lysis of cellular antigens, chemotaxis and anaphylatoxic activity, opsonization and immune adherence, and viral neutralization. During the past twenty years, a large volume of data on the structure, molecular interactions, biological activities, and genetic polymorphisms of the complement glycoproteins has accumulated. Only a brief summary will be attempted here. Various aspects of complement have been reviewed by a large number of investigators, and this summary has been abstracted primarily from reviews by Rapp and Boursos (1970), Muller-Eberhard (1975), Porter and Reid (1978), Fearon and Austen (1980a), Alper (1981), and Whaley and Ferguson (1981).

1. Historical background

Grohmann, in 1884, showed that blood plasma was bactericidal and this observation was confirmed for fresh serum by Buchner in 1889. Attributing the property to serum enzymes, Buchner further noted that it was sensitive to heat and to dialysis against water. Bordet, in papers

published in 1896 and 1898, gave evidence that two components of the immune serum were essential for bacteriolysis. The first, a specific, relatively heat-stable factor, is now known to be antibody. The second, called alexine, was heat-labile and present in immune and non-immune animals.

During the early twentieth century, alexine, or complement, was known to consist of euglobulin, or mid-piece, and pseudoglobulin, or end-piece. Four components were identified, distinguished by euglobulin precipitation, heat-sensitivity, treatment with zymosan or cobra venom, and ammonia-sensitivity. Euglobulin contained the first component; heat-inactivated serum was devoid of components one and two; treatment by zymosan or cobra venom removed the third component, and treatment with ammonia removed the fourth.

While the sequential activity of complement was clearly demonstrated by these early investigators, it was not until 1958 that Rapp demonstrated that the third component was likely to consist of multiple factors. During the same period, between 1954 and 1959, Pillemer and his associates showed that zymosan affected the third component via the protein properdin, and suggested the possible existence of an alternative activating pathway.

2. The pathway

Some twenty proteins are now known to make up the

complement system. The structural features and serum concentrations of these are given in Table I-1. The complete complement pathway is illustrated in Figure I-1. For convenience this has been divided into (a) the classical activation sequence, (b) the late-acting sequence, and (c) the alternative activating sequence.

a. The classical activating sequence

This sequence is initiated when IgM or IgG antibody becomes altered by combination with antigen and acquires the ability to bind C1q of the molecular complex, C1. Binding of two antibody receptor sites by C1q results in the successive enzymatic activation of C1r and C1s, C4 and C2. Two enzymatically-active products of C4 and C2, $\overline{C4b}$ and $\overline{C2a}$, form the classical C3-convertase $\overline{C4b2a}$. This enzyme cleaves C3 to $\overline{C3b}$ leading to production of the C5-convertase $\overline{C4b2a3b}$. The components of these convertases can bind target membranes by means of labile binding sites exposed during enzymatic cleavage.

At least three proteins, C1-inactivator, C4-binding protein, and C3b-inactivator act to regulate this classical activation sequence.

C1-inhibitor binds C1s and C1r at or near their active sites. When one molecule of the C1-molecular complex binds C1-inhibitor, the complex comes apart, releasing smaller complexes which have been shown to consist of two

Table I-1: Proteins of the complement system (1)

Component	Molecular Weight	Number of peptide chains	Serum concentration (µg/ml)	Genetic Variation: Deficiency	Genetic Variation: Polymorphism
a. Classical activating sequence					
C1q	400,000	18(6 x 3)	250	+	-
C1r	90,000	1	100	+	-
C1s	90,000	1	80	+	-
C4	204,000	3	430	+ (2)	+(2)
C2	100,000	1	20	+ (2)	+(2)
C3	190,000	2	1300	+ (3)	+
b. Late-acting components					
C5	185,000	2	75	+	+
C6	128,000	1	60	+	+
C7	121,000	1	60	+	+
C8	153,000	3	80	+	+
C9	79,000	1	50	+	-
c. Alternative activating sequence					
C3b	181,000	2	?	see C3	see C3
B	93,000	1	150	+ (1)	+ (1)
D	25,000	1	2	-	+
P	22,000	4	30	-	-
d. Control proteins					
C1-inhibitor	90,000	1	180	+	+
C4-binding protein	540,000 590,000	8	?	-	+
C3b-inactivator	90,000	2	50	+	-
βlH	150,000	1	300	+	-
S protein	88,000	1	?	-	-
Anaphylatoxin inactivator	300,000	8	?	-	-

(1) Adapted from Hauptmann (1979) and Whaley and Ferguson (1981). (2) Linked to HLA (chromosome 6). (3) Chromosome 19.

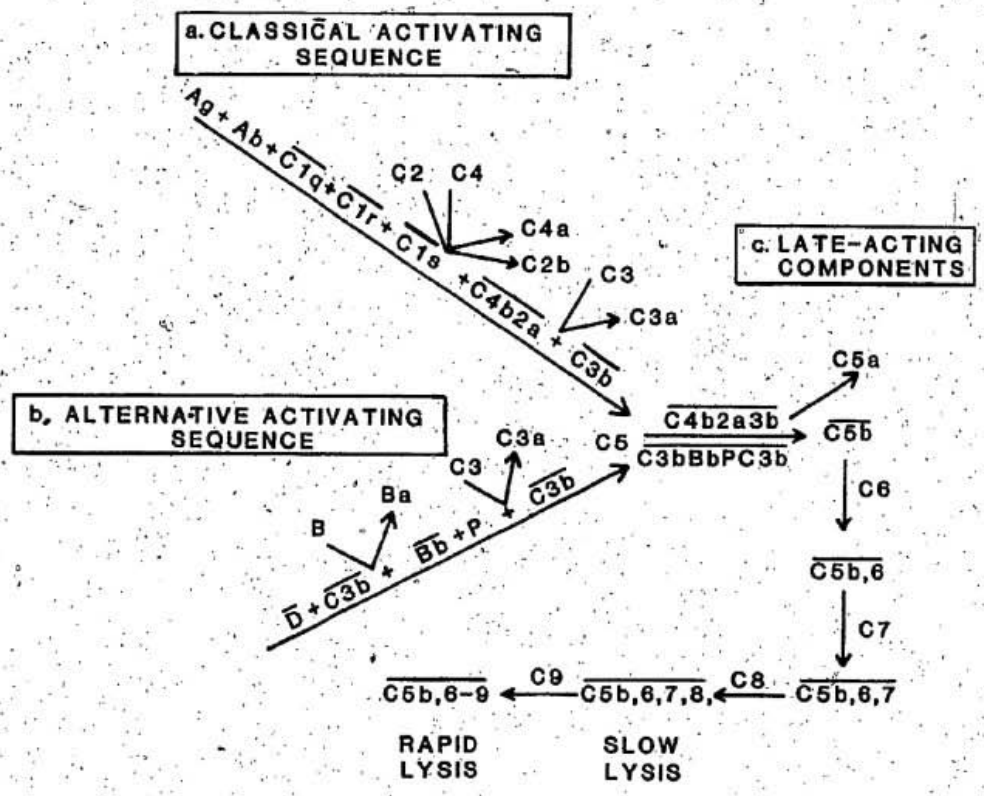


Figure I-1: The complement sequence.

C1-inhibitor molecules associated with one each of C1r and C1s.

C4-binding protein binds C4b forming stable complexes, and in so doing acts as a cofactor for the enzymatic degradation of C4b by C3b-inactivator. C4-binding protein also accelerates the decay of the $\overline{C4b2a}$ complex, probably by displacing C2a. The effect of these proteins on C4b will be discussed in Section B2 of this chapter.

In the absence of cofactors, C3b-inactivator will interact with cell-bound C3b to block participation of C3b in the C5-convertase, to inhibit the binding of C3b-coated particles with C3b receptors on a variety of cells, and to produce a limited cleavage of C3b.

b. The late-acting components

C5 convertase cleaves C5 and leads to complex formation with C6 and C7. The terminal components C8 and C9 attach to the membrane-bound, trimolecular complex, $\overline{C5b,6,7}$. One dimeric complex of $\overline{C5b-9}$ is believed to insert itself into the target membrane leading to a "complement lesion" by disarrangement of the phospholipid bilayer at or near the site of insertion.

The effects of this membrane attack complex are modified by S-protein, which competes for the membrane-binding site thus blocking cytolysis. S-protein has also

been shown to prevent dimerization of the C5-9 complex.

c. The alternative activating sequence.

This system is initiated when Factor B, Factor D, and $\overline{C3b}$ interact so that Factor B is cleaved and activated by Factor D forming the C3 convertase, $\overline{C3bBb}$. This convertase provides the amplification loop of the alternative pathway by generating more $\overline{C3b}$. It is stabilized by the binding of properdin which retards decay of the Bb component.

$\overline{C3b}$ plays two roles in the alternative sequence. It is an integral part of C3 convertase, and it is also essential to the assembly of C5 convertase for which an extra $\overline{C3b}$ molecule is necessary.

Initiation of the alternative pathway is mediated primarily by $\overline{C3b}$, formed by classical or alternative activation or by the action of enzymes such as plasmin. It is prevented from driving the alternate pathway to exhaustion by the action of the control proteins C3b-inactivator and β1H. C3b inactivator interacts with cell-bound C3b to block participation of C3b in the formation of the convertases. β1H controls the pathway on three levels. First, this molecule binds to C3b and prevents binding of Factor B. Second, β1H can displace Bb from $\overline{C3bBb}$ and $\overline{C3bBbP}$, rendering C3b susceptible to C3b-inactivator. Third, β1H is required for complete proteolytic degradation of C3b by C3b-inactivator.

Substances known to stimulate the alternative pathway circumvent the regulatory activities of these proteins. Cobra venom factor is cobra C3b which is resistant to human C3b-inactivator. Nephritic factor, an antibody to determinants formed by C3b and Bb, stabilizes the alternative C3-convertase. It has been shown that binding of β1H to C3b is decreased on surfaces having low sialic acid content, so that formation of $\overline{C3bBb}$ is favoured. It has been suggested that the the complex surface polysaccharides of gram-negative bacteria and yeasts are deficient in sialic acid, and that these surfaces provide micro-environments which are therefore protected from the activities of β1H and C3b-inactivator. In other words, agents which stimulate the alternative pathway simply amplify the pre-existing low-grade turnover of the system.

3. Regulation of complement activation

It is possible to distinguish three levels of control. There is, first, the extreme lability of the binding sites exposed by enzymatic cleavage of C4, C3 and C5. It has been estimated that C4b, C3b and C5b retain the ability to bind cell membranes for less than 100 milliseconds. Second, there is the natural decay of the C3 and C5 convertases. C2a and Bb are known to decay from their respective enzymes. Finally there is the control afforded by the regulator proteins, C1-inhibitor, C3-inactivator, C4-binding protein, and β1H.

4. Biological activities of the complement components

Activation of the late components results in lysis of target cells. In addition, intermediate products are known to produce a variety of biological effects including anaphylatoxic activity and chemotaxis, opsonization and immune adherence, virus neutralization, and modification of immune complexes.

a. Anaphylatoxic activity and chemotaxis

The small products of the enzymatic cleavage of C3 and C5, C3a and C5a, have anaphylatoxic activity. This activity is removed by the action of anaphylatoxic inactivator, carboxypeptidase B. C5a is known to be chemotactic for polymorphonuclear leukocytes, as is Bb, the cleavage product of Factor B.

b. Opsonization and immune adherence

Human erythrocytes, mononuclear phagocytes, polymorphonuclear leukocytes, and B-lymphocytes have receptors for C3b and probably also for C4b. These permit interaction between complement-coated immune complexes and these cells. Interaction with mononuclear phagocytes and polymorphonuclear phagocytes may result in increased phagocytosis. Furthermore, C3b and C4b on bacterial surfaces facilitate attachment to macrophages and increased bacterial killing.

c. Neutralization of viruses

A number of complement components, notably C1, C2, and C4, have been shown to have anti-viral activity. Daniels et al. (1970) have shown, for example, that while neither C1 alone nor C4 alone was able to neutralize IgM-sensitized Herpes simplex virus, C4 was capable of neutralizing virus-IgM that had been exposed to C1.

d. Solubilization of immune complexes

Czop and Nussenzweig (1976) have shown that immune precipitates are solubilized by interaction with the classical complement components and this effect is thought to depend on the intercalation of C3b and C4b into the antigen-antibody lattice. The same group (Miller et al., 1973) have demonstrated that dissociation of cell-bound immune complexes from cell membranes requires an intact alternative pathway.

5. Genetic variation of human complement proteins

Complement proteins show two kinds of genetic variation (Table I-1). The first is deficiency of an individual component, which has been shown for all proteins of the classical activation sequence, for all late-acting proteins, for factor B and properdin of the alternate activation sequence, and for the control proteins C1-inhibitor, C3b-inactivator, and β1H.

The second type of variation is polymorphism due to charge differences, which is usually identified by the use of electrophoresis in agarose gels or by isoelectric focusing. It is likely that charge differences have arisen from single point mutations resulting in amino acid differences.

a. C1

C1q deficiency has been reported by Berkel et al.(1979) and Thompson et al. (1980). In neither case has the genetic basis been firmly established. One propositus, a four-year-old boy, appeared to possess an abnormal, antigenically-deficient C1q which did not activate C1r and C1s. It was suggested that the trait was transmitted as an autosomal dominant.

Eight individuals from four families have been shown to be C1r-deficient. In one family (Lee et al., 1978) this trait seemed to be transmitted by an autosomal recessive gene. All C1r-deficient individuals so far described have half-levels of C1s.

Two families have been identified containing five C1s-deficient individuals (see Tappeiner, 1982). No data are available on the genetic basis of the trait in either case.

b. C2

Approximately 50 cases of complete C2 deficiency have been reported. The deficiency trait, which is consistent with an autosomal recessive mode of inheritance (Klemperer et al., 1966), is linked to the HLA region (Fu et al., 1974). Heterozygous individuals are detected as obligate heterozygotes in C2-deficiency families, or in randomly sampled individuals by quantitative determination of half-levels of C2 antigen or C2 functional activity. By these means, the deficiency trait has been estimated at 1% of the Caucasian population. Evidence from a number of investigators (including Day et al., 1976, Agnello, 1978, and Hauptman et al., 1977) indicates strong linkage disequilibrium between C2-deficiency and the alleles of the MHC haplotype A10(25) B18 Dw2 DR2. By analyzing the segregation of C2-polymorphic variants in C2-deficiency families, Pariser et al. (1978), Marshall et al. (1980a) and Mortensen et al. (1980) were able to show that the deficiency gene is an allele at the structural locus.

Polymorphism of C2 and its linkage to HLA was first described by Hobart and Lachmann (1976a) and further characterized by Alper (1976) and Meo et al., (1977). It is detected by isoelectric focusing of serum, followed by C2-deficient haemolytic overlay. At least four phenotypes have been observed in Caucasians and these are combinations of three allelic products of autosomal codominant genes.

C2*1 (Meo, 1976) or C2*C (Alper, 1976) is the most commonly occurring while the basic (C2*2 or C2*B) and acidic (C2*A1) variants are rare. The frequencies of these variants are for C2*1, 0.96, for C2*2, 0.038 and for C2*A1, 0.001.

c. C4

C4 polymorphism and deficiency will be considered in Sections B and D of this chapter.

d. C3

Deficiency of C3 was first reported by Alper et al. (1969) in heterozygotes with half-normal levels of C3 protein. By tracing the segregation patterns of C3 polymorphic variants in families with heterozygous-deficient individuals, these investigators demonstrated that the deficiency trait was controlled by an allele at the structural locus. A homozygous individual was subsequently observed (Alper et al., 1972a) and six additional completely C3-deficient individuals have since been described.

Polymorphic variants of C3 are detected by agarose gel electrophoresis of whole serum in the presence of Ca++ ions. The most common form in Caucasians is C3*S with the rarer variant C3*F occurring at a frequency of 0.20. A number of very rare variants have been described. All variants are inherited as autosomal codominant traits.

Whitehead et al. (1982), using mouse-human somatic cell hybrids, have localized the structural locus for C3 to human chromosome 19.

e. C5

Rosenfeld et al. (1976) described an individual with no immunochemical or haemolytical C5. Two additional families containing seven C5-deficient individuals have subsequently been observed (Snyderman et al., 1979 and McLean et al. 1981). Heterozygotes have been identified in both families on the basis of half-levels of serum C5, and an autosomal recessive mode of inheritance appears likely.

Although other populations have been tested, separation of serum by isoelectric focusing followed by C5-deficient haemolytic overlay has revealed C5 polymorphism in Melanesians only (Hobart et al, 1981). Two codominant alleles were reported, the common C5*1 with a frequency of 0.93 and the rare C5*2 with a frequency of 0.07.

f. C6

Eight C6-deficient individuals in seven families have been reported (Leddy et al., 1974 and Petersen et al., 1979). Segregation patterns of the polymorphic variants in these families indicate that C6-deficient individuals are homozygotes for a null allele at the structural locus.

Polymorphism is detected by isoelectric focusing and haemolytic overlay containing C6-deficient rabbit serum. Patterns detected are combinations of products of two common codominant alleles, C6*A and C6*B, and a number of rare ones, collectively referred to as C6*R. These alleles occur with relatively similar frequencies in all the major races of man, that is, 0.56-0.61 for C6*A, 0.35-0.38 for C6*B and 0.015-0.06 for C6*R.

g. C7

Deficiencies of this component have been observed in eleven propositi and family studies in most cases show a pattern consistent with autosomal transmission of the deficiency gene. Individuals in two families have proved to be deficient in both C6 and C7. Lachmann et al. (1978a) have described an individual with low serum levels of C6 and C7, in whom the C6 molecule was dysfunctional while the C7 molecule was not. The combined deficiency was shown to be inherited as a single Mendelian trait with heterozygotes having half-normal levels of both proteins. A second family (Glass et al., 1978) with combined C6/C7 deficiency showed no evidence of synthesis of a dysfunctional protein.

Hobart et al. (1978) using isoelectric focusing and C7-deficient haemolytic overlay obtained patterns consistent with the existence of three codominantly-expressed structural alleles for C7. C7*1 is the common allele and C7*2 and C7*3 are rare ones with combined

frequencies of less than 0.01. Through family studies of C6 and C7, these investigators were able to show close linkage between the two loci.

h. C8

Eight individuals with C8 deficiency have been observed since the first report by Petersen et al. in 1976. Familial patterns are consistent with an autosomal recessive mode of inheritance and Raum et al. (1979a) have shown that the C8-deficiency gene is an allele at the structural locus. Two types of C8 deficiency are now recognized. In type I, the α-γ chains of the three-chain C8 molecule are absent. In type II, only the β-chain is absent.

Raum et al. (1979a) have used isoelectric focusing and C8-deficient overlay to identify patterns consistent with the existence of three codominant alleles. The most common is C8*A, with a frequency of approximately 0.7 in white, black, and oriental populations. C8*B has a frequency of approximately 0.3, while C8*A1 occurs mainly in black populations with a frequency of 0.03-0.05. This polymorphism is thought to reside on the α-γ portion of C8. Marcus et al. (1982) used type II C8-deficient overlay to detect a second polymorphism on the β-chain of the C8 molecule. These investigators have stated that β and α-γ alleles segregate independently in families, suggesting

that C8 is produced at two separate, unlinked genetic loci.

i. C9

Identification of C9-deficient individuals has been hampered by the fact that lysis will proceed slowly in the absence of C9. Three unrelated individuals with C9 deficiency have been detected, however, whose serum predictably did exhibit both haemolytic and bacteriolytic activities, but at much slower rates than normal. Family studies (Lint et al., 1980) indicate half-normal levels of C9 in heterozygotes and an autosomal mode of transmission of the deficiency gene.

j. Factor B

No completely Factor B-deficient individual has been described. Hauptmann (1976) and O'Neill (1982a) have described rare polymorphic variants with no functional activity, and a family has been described (Suciu-Foca et al., 1980) in which BF patterns in three siblings are consistent with transmission of a BF null allele at the structural locus.

BF polymorphism was first described by Alper et al. (1972b) and was subsequently shown to be HLA-linked (Allen et al., 1974). Variants are detected by immunofixation electrophoresis with specific anti-Factor B. Two common allelic variants, F and S, two rare ones, F1 and S1, and at least seven very rare variants have been detected.

Population studies have shown significant associations between alleles at the BF locus and HLA alleles, particularly HLA-B and HLA-DR. These associations will be considered in Section C4, this chapter.

k. Factor D.

Hobart and Lachmann (see 1976b) using isoelectric focusing and D-deficient haemolytic overlay found a variant of Factor D in three individuals of West African origin. They observed no variants in plasmas from 115 British and 25 Indians, and noted that Factor D, unlike other components examined, gave a single-banded pattern in isoelectric focusing.

l. C1-inhibitor

Donaldson and Evans (1963) demonstrated a genetically-determined deficiency of C1-inhibitor in patients with angioneurotic edema, a genetic disease which is transmitted as an autosomal dominant. There are two forms of this disease. In approximately 80% of affected families, individuals with the disorder have low serum levels of normal C1-inhibitor. In the remaining families, affected individuals have normal or high levels of a dysfunctional protein. Comparison of dysfunctional proteins produced by patients of the second type shows that these vary in electrophoretic mobility, and in their ability to bind and inhibit C1s (Rosen et al., 1971). It seems likely

that the disease state can be produced by a number of different mutant genes.

m. C3b-inactivator

At least two cases of C3b-inactivator deficiency have been observed (Alper et al., 1970 and Thompson et al., 1977) and, as expected, the consequence is hypercatabolism of Factor B and more especially C3. Although presumed heterozygotes for this deficiency have half-normal levels of the protein, they have normal levels of C3 and Factor B. The precise genetic control of this deficiency is still uncertain.

n. C4-binding protein

Rodriguez de Cordoba et al. (1982) have demonstrated polymorphism of C4 binding protein by the technique of isoelectric focusing in agarose followed by immunofixation with polyclonal and monoclonal antibodies. They found two banding patterns with frequencies of 98% and 2%.

o. β1H-globulin

Thompson and Winterborn (1981) have described a case of hypocomplementaemia in an Asian male infant due to a deficiency of β1H. They found very low levels of β1H in a clinically healthy sibling, and half-levels in the parents who were first cousins. A genetic defect therefore appears likely.

B. The fourth complement component in man

Gordon et al., in 1926, reported that a lipase preparation suspended in ammonia-containing buffer could abolish the haemolytic activity of human serum without affecting the other three components known at the time. These investigators subsequently showed that there was a fourth component, characterized by its sensitivity, not to lipase, but to ammonia and other primary amines, by its relative heat-stability, and by its absence from the euglobulin fraction of plasma. Muller-Eberhard and Biro (1963) localized haemolytic activity of the fourth component to β1E-globulin, an immunologically and electrophoretically pure serum protein of the pseudo-globulin fraction with a sedimentation coefficient of 10S.

1. Structure of C4

Data from various laboratories (Schreiber and Muller-Eberhard, 1974; Gigli et al., 1977) indicate that the C4 molecule consists of three disulphide-linked polypeptide chains, α, β, and γ, with molecular weights of 93,000, 78,000, and 33,000, respectively (Figure I-2). Carbohydrate constitutes approximately 7% of total protein weight. Oligosaccharides are present on both α- and β-chains, with the α-chain containing several oligosaccharide moieties, and the β-chain containing a high-mannose unit.

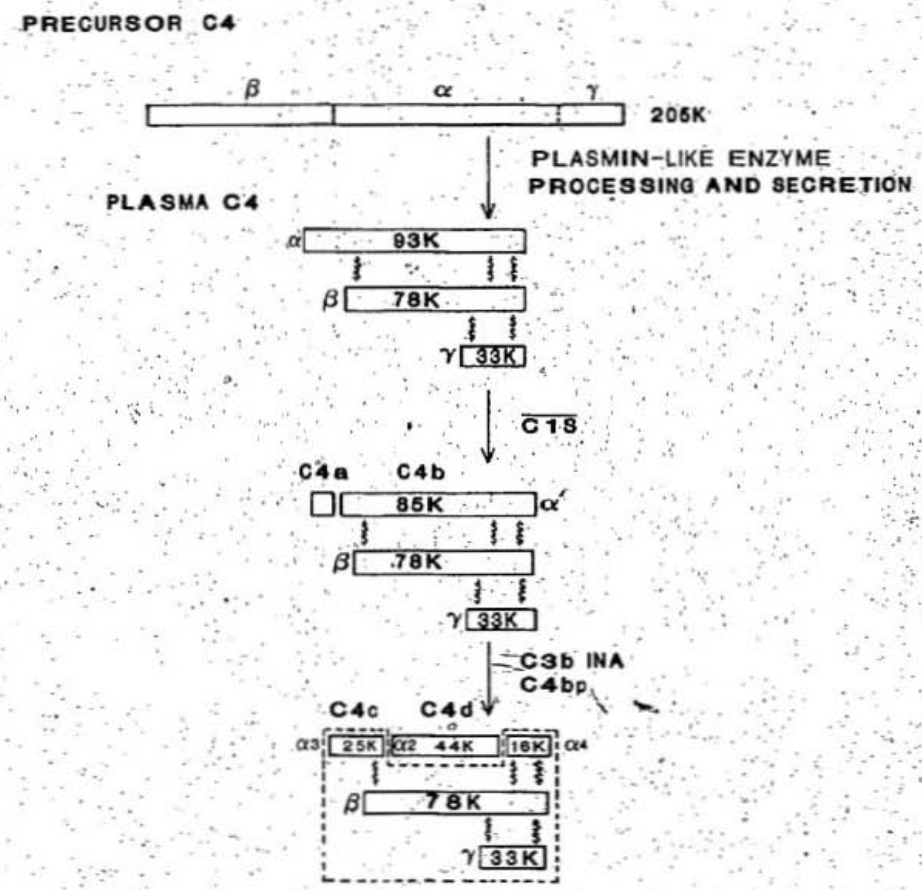


Figure I-2: Proteolytic degradation of the C4 molecule.

There is minimal, if any, carbohydrate on the γ-chain (Gigli et al., 1977).

2. Function of C4

C4 is converted to its functional form when C1s, generated by the interaction of C1 with an immune complex, produces conformational alterations in the native molecule and cleavage of a single peptide bond between residues 77 and 78 of the α-chain. An activation peptide, C4a (m.w. 9000), and a major cleavage fragment, C4b (m.w. 186,000) are produced.

a. C4a

Gorski et al. (1979) have shown that C4a is biologically similar to, but less potent than, the C3a and C5a anaphylatoxins in inducing ileal contractions and skin wheal and flare. Electrophoresis at pH 8.6 indicates an isoelectric point for C4a very near that of C3a, and spectral analysis implies that the molecule, in common with C3a and C5a, has a part alpha-helical configuration. Partial sequencing data show homology between the three anaphylatoxins.

b. C4b

Conformational changes induced by cleavage of C4a result in acquisition by C4b of four interactive sites. There is a labile-binding site by which C4b binds

covalently to the activating surface, most likely through the Fd region of antigen-bound IgG (Campbell et al., 1980). There are stable sites for interaction with C4-binding protein and consequent degradation by C3b-inactivator. There is a stable site for C2 producing the $\overline{C4bC2a}$ complex, the classical C3-convertase. Finally, there is a stable site by which C4b attaches to a variety of circulating cells via their immune adherence receptors.

i. The labile binding site

The chemical nature of the labile binding site has recently been substantially clarified. When native C4 is treated with a primary amine (Gorski and Howard, 1980, Janatova and Tack, 1981) the product is a C4b-like molecule which, though retaining ability to function in the fluid-phase C3-convertase, has lost its ability to bind target surfaces and is therefore haemolytically inactive. Such treatment leads to liberation in the C4 molecule of a free sulphydryl group and a reactive acyl group which is attacked by the nitrogen nucleophile (Janatova and Tack, 1981). It is now believed that C4 possesses an internal thioester bond which, when the molecule is cleaved to C4b, produces a reactive acyl group through which it forms an ester or amide link with the target surface (Law et al., 1980). The free thiol group so formed has been localized to a cysteine residue on the C4d fragment (Figure I-2) of the α-chain and the reactive acyl group to a glutamic acid three residues removed from this cysteine (Campbell et al.,

1981).

It has also been shown that haemolytic inactivation of C4 and concomitant sulphydryl exposure can also be produced by low concentrations of chaotropic ions or by slow freezing and thawing (von Zabern et al., 1981). In this case, the nucleophile is thought to be water. Scission of the thioester bond can lead therefore to production of haemolytically-active C4b, through covalent attachment by transacylation to the target surface, or, by hydrolysis by water, to production of haemolytically-inactive C4b in the fluid phase.

ii. Proteolysis of C4b

C4b is degraded by the action of C3b-inactivator (Cooper, 1975) and its high molecular weight cofactor, C4-binding protein (Scharfstein et al., 1978). C4-binding protein appears to be essential for proteolysis of fluid-phase C4b (Fujita et al., 1978), and, while not an absolute requirement for degradation of cell-bound C4b, it greatly accelerates the process (Gigli et al., 1979). Preliminary evidence suggests that C4-binding protein may bind C4b by a transacylation mechanism similar to that described for the attachment of C4b to target surfaces (Villiers et al., 1982).

C3b-inactivator cleaves C4b at two sites on the α'-chain, producing C4d (m.w. 44,000 - 46,000) which is the $\alpha 2$-fragment, and two smaller fragments, $\alpha 3$ (m.w. 25,000)

and α4 (m.w. 16,000 - 17,000). The α3- and α4-chains remain associated with the β- and γ-chains forming C4c (Figure I-2). Data from two laboratories (Nagasawa et al., 1980 and von Zabern et al., 1982) indicate that this proteolysis is a two-stage process in which the smaller α4 is first cleaved from the remaining α3-C4d fragment, followed by cleavage of this latter fragment, releasing C4d. Furthermore, amino acid sequencing of the α-chain products so formed (Press and Gagnon, 1981) and SDS-electrophoretic separation of the cleavage products of inactive C4 molecules (von Zabern et al., 1982) provide evidence consistent with the order, α3, α2 (C4d), α4, of the fragments in the intact α'-chain of C4b.

iii. Interaction with C2

When activated by C1s, C2 is cleaved into C2a and C2b (Polley and Muller-Eberhard, 1968) and the fragments reversibly bind to C4, possibly via the N-terminal C2b part of the molecule (Kerr, 1980). C2a and C4b interact to form the C3-convertase, $\overline{C4b2a}$. Decay of the convertase occurs because C2a, which carries the enzymatic site for $\overline{C4b2a}$, decays from the complex (Nagasawa and Stroud, 1977).

Since the ability to bind C2a is unaffected by treatment of C4 with amines and chaotropic ions (von Zabern et al., 1981) the site for C2a binding is assumed to be spatially removed from the labile binding site. Furthermore, since C2a protects C4b from the activity of

C3b-inactivator (Cooper, 1975), the receptor for C2a is likely to be proximate to a C3b-inactivator cleavage site.

iv. Immune adherence

Cooper (1969) was able to show that C4b, as EAC14, had the ability to produce immune adherence when human erythrocytes were used as indicator particles. This activity was abolished by enzyme-treatment of the human erythrocytes. Clark and Klebanoff (1978) found that opsonization of zymosan by serum was markedly impaired when C4-deficient serum was used. Ferrone et al. (1976) have suggested that lymphocyte-bound C4 appears to be necessary for mixed lymphocyte responses and mitogenic responses.

From studies on the effect of C3b-inactivator on C4b, Cooper (1975) was able to show that the stable binding site by which C4b attaches to receptor-bearing cells is spatially removed from the C2 binding site, since attachment of C2a to C4b has no effect on immune adherence activity. Furthermore, cleavage of cell-bound C4b by C3b-inactivator resulted in concomitant loss of immune adherence activity and loss of C4c, implying that the site for immune adherence is in this fragment.

3. Biosynthesis of C4

Available evidence indicates that the chief extra-hepatic sites of C4 synthesis are the macrophages (see Colten, 1982).

Hall and Colten (1977) in cell-free synthetic studies, identified a single chain protein, immunochemically related to guinea pig C4, which they suggested was a precursor of the native C4 molecule. It was subsequently shown (Gorski and Muller-Eberhard, 1978; Gigli, 1978) that human plasma contained a small amount of single-chain C4 which, while antigenically identical to the three-chain molecule, was haemolytically inactive. Parker at al. (1979) and Fey et al. (1980), using pulse-chase experiments on cultured peritoneal exudate macrophages, showed that single-chain precursor C4 (pro-C4) disappears from cytoplasmic extracts in a reciprocal manner to the appearance of extracellular mature protein. No pools of extracellular precursor or of intracellular mature protein were found, indicating that processing took place in association with cells and the secretory process.

By comparing amino acid sequences obtained from pro-C4 and C4 subunit chains, a number of investigators (Goldberger et al., 1980a; Parker et al., 1980; Karp et al., 1981) have established that the order of C4 subunits in pro-C4 is $\beta-\alpha-\gamma$. The production of α, β, and γ-chains by plasmin treatment of pro-C4 implicates a plasmin-like enzyme in the post-translational processing of the molecule (Goldberger and Colten, 1980b).

Glycosylation has also been shown to be essential to intracellular processing. Matthews et al. (1982) have shown

that pro-C4 is glycosylated post-translationally and inhibition of glycosylation decreases the rate of C4 secretion and increases the rate of intracellular C4 catabolism but does not affect the haemolytic activity of the secreted underglycosylated native C4.

A recent report by Chan et al. (1983) suggests that extracellular processing also plays a role in the production of native circulating C4. These investigators have described a secreted form of C4, comprising up to 8% of plasma C4, and the predominant form secreted by a human hepatoma cell line. The α-chain of this secreted form was 5000 daltons heavier than that of the major plasma form and this difference was not removed by deglycosylation.

4. Polymorphism of C4

a. The genetic model

Electrophoretic polymorphism of the fourth component in man was first described by Rosenfeld et al., (1969) using the technique of antigen-antibody crossed electrophoresis on human plasmas. They found five complex patterns which were combinations of three subtypes, A1, A, and C. The genetic control of these variants was unclear but it was suggested that A and A1 might be allelic forms at a structural locus separate from that of subtype Ø. Bach et al. (1971) used the same technique to examine paired

maternal and cord plasmas. By sampling fathers of those pairs which differed in C4 patterns, it was shown that foetal C4 patterns were indeed combinations of parental ones. Although the exact genetic mechanism was not clarified, the suggestion of two structural loci was again made:

Teisberg et al. (1976) using immunofixation electrophoresis found three common electrophoretic patterns: F, a "short" anodal variant, S, a "short" cathodal variant, and FS, extending through the F and S regions. These products were thought to be derived from alleles at a single structural locus, FS being the heterozygous product. Mauff et al. (1978) introduced a modified buffer system, obtained similar results, and described an additional variant. Subsequent reports added more rare variants and a "long" variant similar in position and pattern to FS but whose F and S components did not segregate in families according to a one-locus pattern (Teisberg et al., 1977).

O'Neill et al. (1978) made the important observation that Chido and Rodgers antigens, known to be non-allelic, were antigenic determinants of C4. The fast-moving C4F carried the Rodgers antigen and the slow-moving C4S carried Chido. It was suggested that a two-locus genetic model best fit the array of phenotypes. These investigators postulated a common null allele at each locus such that F variants

(Ch-Rg+) were genotyped Fso/Fso, S variants (Ch+Rg-) foS/foS, and "long" variants, FS (Ch+Rg+), carried null alleles at one or two or no loci. The homozygous null condition was C4 deficient and rare.

That there are two C4 genes on some chromosomes was verified by Olaisen et al. (1979b). A high-titre anti-Ch antibody was used to remove the S component only from plasmas of individuals known to carry FS haplotypes, that is, from individuals who had inherited the FS haplotype from one parent and either F or S from the other. Loci on a single FS haplotype in these individuals were thus shown to direct the production of two antigenically distinct sets of C4 molecules.

Using immunofixation electrophoresis and untreated EDTA or heparin plasmas, FS, F, S, and F1 (fast F) patterns could be clearly distinguished but interpretation of many variants was complicated by combinations of these common variants with rarer ones which had intermediate mobilities and by the difficulty of making genotypic assignments of the phenotype FS.

The method of Awdeh et al. (1979, 1980) has helped to solve the first problem. Pre-treating EDTA-plasma with neuraminidase resulted in modified electrophoretic mobility such that the F variants (called A by these investigators) moved faster relative to the S variants (called B) and the whole pattern was more spread out and more clearly banded

on the electrophoretic plate. Intermediate variants were more easily identified and small differences in the whole pattern more readily seen. Since 1980, two naming systems have existed. The letters F, S (and F1, D, I, M, etc.) have been used by Teisberg et al. (1980a), Petersen et al., (1979), Rittner et al. (1980a) and O'Neill et al. (1980a) to identify, in most cases, variants obtained from untreated samples. The letters A (A3, A4, A2, AQO) and B (B2, B1, BQO) have been used by Awdeh and Alper (1980a), Bruun-Petersen et al. (1981), Roos et al. (1982a), and O'Neill et al. (1982b) to identify variants seen after pretreatment with neuraminidase. These variants are described in the next section.

The problem of assigning genotypes to FS phenotypes has been less easily resolved. O'Neill and Dupont (1979a) described three FS types: FS where F and S stained with equal intensity after immunofixation; F'S where the F component was heavier; FS' where the S component stained more heavily. Where the S or F component was weaker relative to the other component in a haplotype, it was assumed to be heterozygous null. Typing unrelated individuals in this manner gave observed phenotype frequencies which did not fit the Hardy-Weinberg equilibrium. Petersen et al. (1979) made similar assumptions and got similar results. In both cases, there was an excess of observed FS phenotypes over expected and a decrease in F'S and FS', indicating likely mistyping or

misinterpretation of the genotypes which FS phenotypes represented.

Awdeh et al. (1979) proposed the use of antigen-antibody crossed electrophoresis (AACE) as a partial solution to this problem. AACE gave somewhat more quantitative indication of relative F (or A) and S (or B) dose in a phenotype. This method still did not distinguish FS/FS from foS/Fso. There were, moreover, no data presented by these investigators on the relative quantity of C4 produced per F and S gene.

The exact genetic mechanism by which C4 is controlled is still unclear. Awdeh and Alper (1980a), O'Neill et al. (1978), and others favour the interpretation that the possession of two loci per chromosome is universal in man and that all "short" haplotypes carry null alleles at one locus. There are two loci in the mouse (Roos et al., 1978) which may be comparable. A recent report (Granados et al., 1982) indicates the likely existence of two loci in other non-human primates. Olaisen et al. (1979b) have postulated that the precise number of C4 genes per chromosome may vary and that the number an individual possesses may be a polymorphism in itself. These investigators accept that in "long" haplotypes there are two genes producing two protein products. They do not accept that "short" haplotypes are necessarily duplicated. Null genes cannot be demonstrated directly. C4 double-null haplotypes must be rare. Only one

locus has been demonstrated in guinea pigs (Bitter-Suermann et al., 1977). Recent reports (Bruun-Petersen et al., 1982, and work described in this thesis) of human haplotypes bearing two A or two B genes indicate that there may be more than two genes per chromosome.

These are different interpretations of similar data. It is likely that arguments will be resolved only when details of the organization of C4 DNA become available. In this regard, Carroll and Porter (1983) have recently reported cloning of a human complement C4 gene in which a cDNA probe specific for the amino acid sequence of C4d was used to identify C4 DNA sequences in a human genomic library. Only one gene was identified in this preliminary investigation but the existence of a second was not excluded, and no data were given on the C4 phenotype of the library donor. Two single amino acid substitutions in the C4d sequence were confirmed and a third described, interpreted by the authors as allelic or locus variation.

b. The electrophoretic patterns

Although different naming systems and somewhat different techniques have been used, patterns can be compared. Table I-2 lists gene products reported by different laboratories arranged so that those likely to be identical are listed in the same column. The most common allelic product at one locus is A3 (or F or F3) with frequency estimates ranging from .66 to .81. The products

Table I-2 : Comparison of proposed C4 gene products reported by various investigators. Gene products entered in the same column (except where indicated by parentheses) are assumed identical.

Investigator	C4 gene products											
	F or A products								S or B products			
1*				F				fo		S		so
2*	F^X			F				fo		S		so
3*	D			F	I				M	S	(T)	
4*	F1			F	(F^V)			fo	(S^V)	S	S1	so
5*	F1			F	I			fo	M	S	S1	so
6*	F1			F	I					S	(SR)	
7*	D1		D2	F	I				M	S		
8*	A6		A4	A3	A2		A1	AQO	B2	B1		BQO
9*	A6	A5	A4	A3	A2	A7	A1	AQO	B2	B1		BQO
10*	F6	F5	F4	F3	(F2)	F7	F1		S2	S1		

* 1 O'Neill et al. (1978) 2 Petersen et al. (1979)
3 Olaisen et al. (1980) 4 O'Neill (1981)
5 Rittner et al. (1980a) 6 Greiner et al. (1980)
7 Teisberg et al. (1980b) 8 Awdeh and Alper (1980a)
9 Alper, pers. comm. (1980) 10 Bruun-Petersen et al. (1981)

of other alleles at this locus, in order of decreasing mobility, are A6 (D1, F1, Fx, or F6) at .04 to .07, A5 at .005, A4 (or D2) at .03 to .06, A2 (or I) at .08 to .09, and the null allele (AQO or fo) estimated at .09 to .19. The most frequent product at the second locus is B1 (or S or S1) with an estimated frequency of .64 to .76. The next most common allelic products are B2 (or M or Sv) at .11 to .19 and the null BQO (or so) at .14 to .16. Rare allotypes S4, S5, T, S1, and SR have also been reported.

The approximate electrophoretic positions of these products are given in Figures I-3 and I-4. Figure I-3 shows some of the gene products obtained using untreated plasma samples. Figure I-4, adapted from one prepared for the Complement Genetics Workshop, 1982, shows all common and rare gene products obtained from desialated samples by four groups of investigators. Many of the rare allelic products shown in the latter figure have been described in one or a few families only and are as yet unpublished.

The distinction between A and B (or F and S) allotypes was originally made on the basis of electrophoretic mobility, A allotypes being anodal to B. With the discovery of intermediate products, this distinction was less useful, particularly when using untreated samples. Awdeh and Alper (1980a) observed that after neuraminidase treatment, the A products were inactive or weakly active functionally, and did not develop when C4-deficient haemolytic overlays were

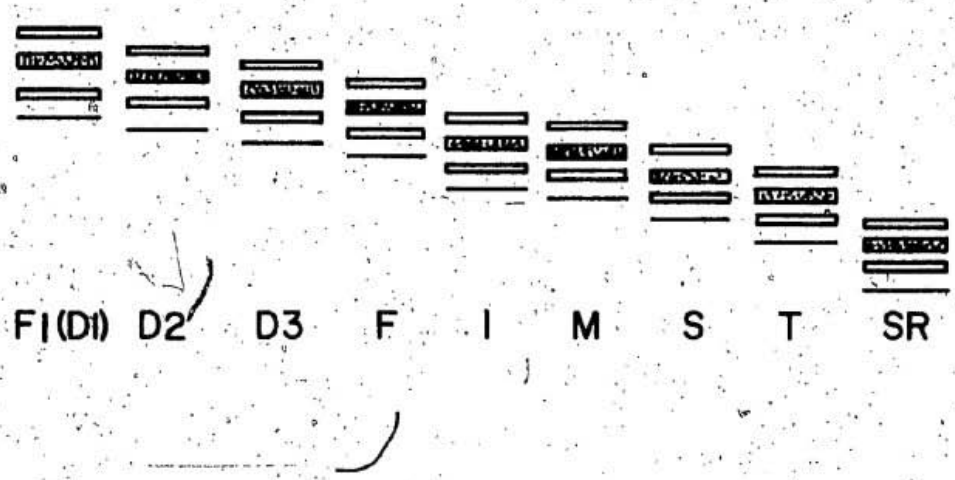


Figure I-3: Allotypic patterns of native C4 (presented to the Complement Genetics Workshop, Boston, 1982).

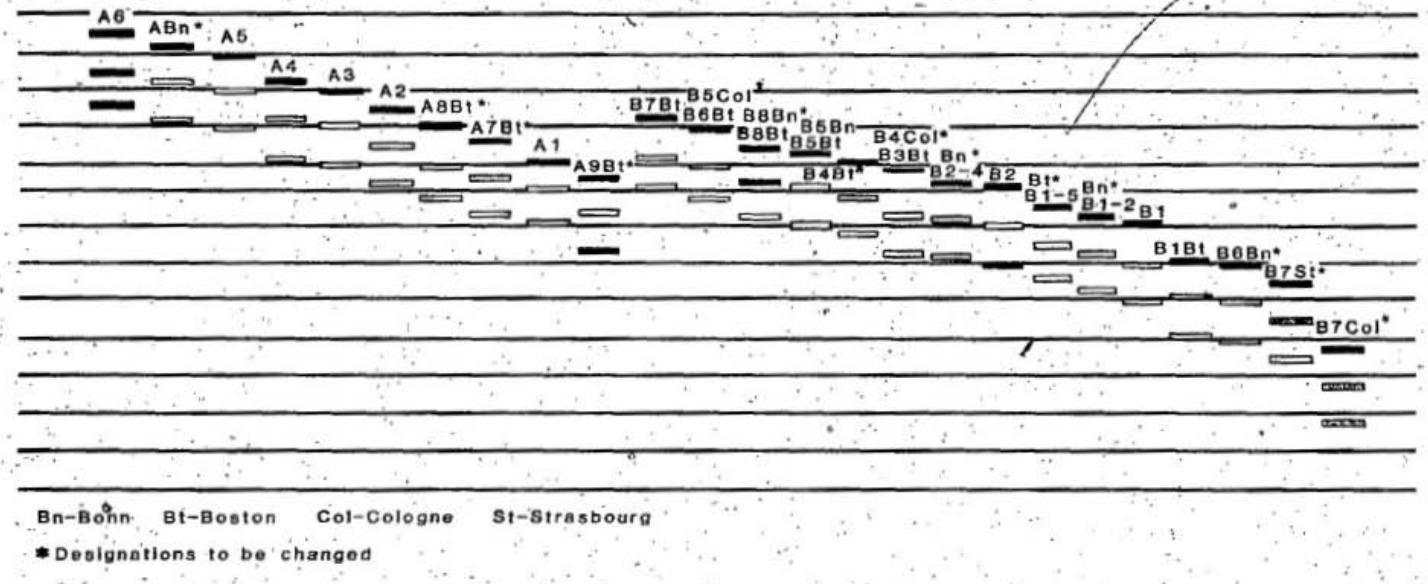


Figure I-4: Allotypic patterns of desialized C4. (Presented to the Complement Genetics Workshop, Boston, 1982.)

used to visualize the C4 patterns. B-locus variants retained full activity. The assignment of A and B identity has therefore been made primarily on the basis of functional activity (Alper, pers. comm., 1980).

Figure I-5 shows schematic representations of the haplotype products obtained by two investigators using (a) untreated and (b) neuraminidase-treated samples. Table I-3 shows haplotype products reported by four laboratories arranged so that haplotypes assumed to be identical are in the same column. Not all haplotypes are shown. A3B1 (FS or F3S1) is the most frequent haplotype with frequency estimates ranging from .47 to .74. The half-null A3BQ0 and AQ0B1 ("short" F and S, or Fso and foS) are next with .14 to .22 and .07 to .17, respectively.

Comparing haplotype frequencies (Table I-3) with gene frequencies (Table I-2) reported by the same laboratory indicates that some haplotypes, A4B2 from Awdeh and Alper (1980a), for example, occur more frequently than expected from the corresponding gene frequencies. Others, like A6BQ0 (or D1) have never been reported. Finally, there has been no report of recombination between alleles of the C4A and C4B loci.

c. Chido and Rodgers antigens

Chido and Rodgers were first defined as red cell antigens through the use of patients whose blood proved

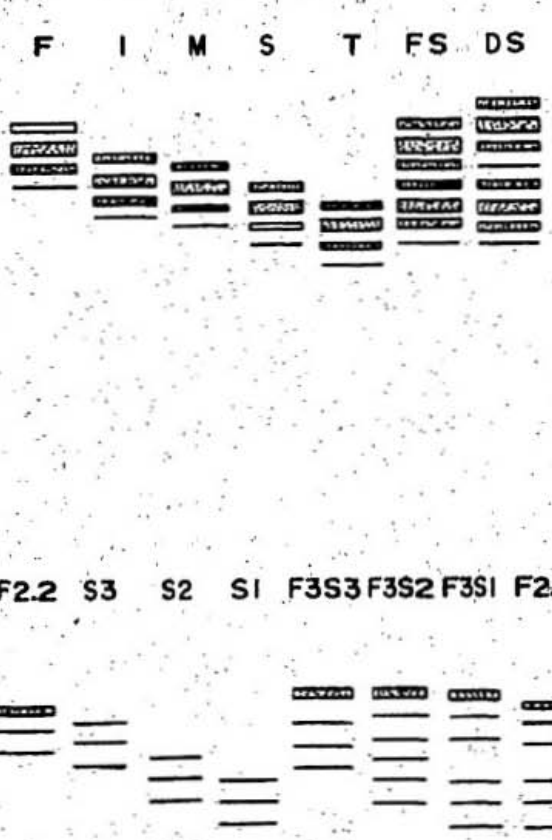


Figure I-5: Schematic representation of C4 haplotype products obtained from (a) native plasma (after Teisberg et al., 1980a) and (b) from neuraminidase-treated plasma (after Bruun-Petersen et al., 1981).

Table I-3: Comparison of most commonly occurring C4 haplotypes reported by various investigators. Haplotypes in the same column are assumed identical except where indicated by parentheses.

Investigator	C4 haplotype								
1*	DS	F	FS					M	S
2*	$F^{X}S$	Fso	FS						foS
3*		Fso	FS						foS
4*	D1S	F	FS					M	S
5*	A6B1	A3BQ0	A3B1		A2B1	A3B2	A4B2	AQOB2	AQOB2
6*	F6B1	F3	F3S1	(F2)	(F2S1)	F3S2		S2	S1
7*	D1S	F	FS	I	IS	FM	D2M	M	S
8*	F1S	Fso	FS					(S^{V})	foS

* 1 Olaisen et al. (1979a) 2 Petersen et al. (1979)
3 Awdeh et al. (1979) 4 Bruun-Petersen and Lamm (1980)
5 Awdeh et al. (1980a) 6 Bruun-Petersen et al. (1981)
7 Nordhagen et al. (1981) 8 O'Neill (1981)

difficult to match for transfusion (Harris et al., 1967, Longster and Giles, 1976). Anti-Chido sera were described as "nebulous" because of the difficulty in distinguishing weak positive from negative results in direct agglutination tests. This difficulty was overcome by the discovery that plasma from individuals whose red cells were weakly or strongly Chido positive inhibited the reaction between anti-Chido and Chido positive red cells (Middleton, 1972a). Thus Chido substance was reliably identified in plasma and proved to be present in the plasmas of some 98% of the Caucasoid population (Middleton and Crookston, 1972b).

Rodgers antigen was also found in plasma with a frequency of approximately 97% in Caucasoids (Longster and Giles, 1976). A small fraction (3%) of Rodgers positive individuals were shown to be Rodgers "partial inhibitors", whose plasmas weakly inhibited the reaction between anti-Rodgers and Rodgers positive red cells.

The genetics of these antigens proved difficult to characterize. Both Ch and Rg traits were genetically controlled and both were coded by genes in the major histocompatibility complex, situated close to HLA-B (Middleton et al., 1974; Giles et al., 1976). Population studies showed the absence of each antigen to be associated with products of particular HLA-B alleles. While low frequencies of Ch- and Rg- phenotypes implied that the traits were not antithetical, no Ch-Rg- individuals were

observed.

A major breakthrough in interpreting these data came with O'Neill's observation that Ch and Rg substances were separate antigenic determinants on two subpopulations of C4 molecules. A C4 deficient individual was shown to be Ch-Rg-. Frequencies of Ch- and Rg- individuals reflected frequencies of C4 Fso and foS individuals. Associations with particular MHC alleles reflected associations between these alleles and C4 (O'Neill et al., 1978).

Since these discoveries, interest in Ch and Rg antigens has been directed towards three general areas, namely (i) the relationship between plasma and red cell Ch/Rg/C4, (ii) localization of Ch and Rg determinants on C4 molecules, and (iii) characterization of the Ch and Rg antigens of C4 polymorphic variants.

(i). The relationship between plasma and red cell Ch/Rg/C4

It is generally accepted that the presence of Chido and Rodgers antigens on human red cells is the result of uptake of C4b from plasma after complement activation, a process similar to the "in vitro" coating of red cells by the activation of autologous serum with low ionic strength sucrose (Tilley et al., 1978). Rosenfield and Jagathambal (1978) have shown that normal red cells carry low and variable levels of C4d, acquired either "in vivo" or "in vitro". "In vitro" uptake could be the outcome of complement activation by cold haemagglutins known to be

present at low levels in many sera (Nordhagen et al., 1979). Chido antigen on red cells is known to increase on storage. The individual variation in red cell Ch and Rg activity which is well known, may reflect differences in the susceptibility of different plasmas to the activation of complement (Rosenfield and Jagathambal, 1978), or individual differences in the quantity of plasma Rg, Ch, and/or C4 (Tilley et al., 1978).

Nordhagen et al. (1979) have found that the C4/Ch/Rg on cells coated "in vitro" by low ionic strength sucrose treatment is resistant to enzymatic attack, whereas C4/Ch/Rg on normal untreated cells is readily destroyed by enzymes. Activated C4 has a labile binding site (in the C4d fragment) by which C4b attaches covalently to target cell surfaces and a stable site (in the C4c fragment) by which C4b attaches to the C3b/C4b immune adherence receptor of a variety of cells. Binding through the latter is susceptible to enzymatic attack, through the former is not. The suggestion has been made that two mechanisms of uptake of C4/Ch/Rg from plasma may be possible; one is via C4d and is enzyme-resistant while the other, immune adherence, is via C4c and is enzyme-susceptible (Nordhagen et al., 1979).

(ii). The localization of Chido and Rodgers determinants on C4 molecules

It has been shown, through the use of anti-sera to Ch, Rg, and to activation fragments of C4 (C4c and C4d), that

red cells coated with Ch and Rg antigens by treatment with low ionic strength sucrose carry C4d (Tilley et al.,1978, Nordhagen et al.,1979). A recent report, however, has challenged these findings. Chu et al. (1982) have found that cells coated in this manner with Ch/Rg at 4° C carry C4c and C4d and cells treated at 37°C carry C4c only. They have further demonstrated that the reactions between anti-Ch and anti-Rg and the Rg and Ch antigens on these cells could be neutralized by purified whole C4, C4d, C4c, and by the α4 fragment of C4c. Ch and Rg antisera therefore appear to be polyspecific, identifying determinants on C4d (α2 chain of C4) and on C4c (α4 chain) as well. Since immune adherence is mediated through the C4c fragment of C4, these data may be taken as further evidence that this process plays a role in the attachment of Ch and Rg antigens to red cells.

Mevag et al. (1981), using two-dimensional electrophoresis of C4 α-, β-, and γ-chains, have localized the charge differences responsible for C4 electrophoretic polymorphism to the α-chain of the C4 molecule. Lundwall et al. (1982) have shown that fragments resembling C4c and C4d are liberated from C4 by treatment with trypsin. Trypsin-C4d carries the labile-binding site with cysteine at position 31 and the methylamine-binding residue at residue 34. They found four forms of trypsin-C4d differing in charge and size but very similar in chemical composition as judged by reaction with xenoantibody and overall amino acid

composition. The size difference parallelled the presence of Rodgers and Chido antigens, such that tryp-C4d of 30,000 m.w. carried the Rodgers antigen and tryp-C4d of 28,000 m.w. carried Chido. Furthermore, evidence for an extra cysteinyl residue in the 28,000 fragments and an increased ability to bind methylamine and iodoacetic acid was reported.

(iii). Characterization of the Ch and Rg antigens of C4 polymorphic variants.

The introduction of neuraminidase treatment of plasma samples has uncovered an impressive degree of polymorphism of C4 proteins. The distinction between A (or F) and B (or S) gene products has not been made primarily on the basis of the presence or absence of Ch and Rg antigens, but rather on the bases of electrophoretic mobility, functional haemolytic activity, and segregation in families assuming two linked genes per haplotype. Generally speaking, however, C4*A variants have been shown to carry the Rg determinants. These include D1, D2, F, and I gene products described by Teisberg et al. (1980a) and the equivalent A6, A4, A3, and A2 of Awdeh and Alper (1980a). The C4*B (or S) variants carry Chido. Olaisen et al. (1979b) were able to remove S and M products with anti-Ch from known haplotype products MS, FS, M, S, and Awdeh and Alper (1980a) and others have stated that the B1 and B2 variants are Ch+Rg-.

There are five reports of variants which do not

completely fit this general pattern:

(a). Mauff et al.(1978) described a fast F variant called F1. This variant has been observed by others and identified variously as Fx, D1, F6 and A6. In all cases it occurs on a F1S (or D1S or A6B1) haplotype. The variant is functionally inactive on many (O'Neill et al., 1980a) or all (Teisberg et al., 1980b) haplotypes. O'Neill et al. have reported that this variant has always typed Rg "partial inhibitor", whereas others have found it to be Rg positive.

(b). Bruun-Petersen and Lamm (1980) have described an intermediate variant, a fast S, called G, usually found on an FG haplotype. The product of this allele was typed as Rg "weak inhibitor".

(c). By careful titration of anti-Ch sera, Nordhagen et al. (1980) have found individuals who were Ch "partial inhibitors" in the agglutination test. This trait was strongly associated with the possession of the C4M (B2) haplotype product.

(d). Nordhagen et al. (1981) found 10 informative Rg "partial inhibitor" haplotypes, of which five carried the C4 haplotype FI, three carried I, one carried IM and one FS. F and I are alleles of the F (or A) locus, and the haplotype FI represents the very unusual situation where two F genes are carried on the same chromosome. All FI haplotypes and three out of four I haplotypes found by these investigators were Rg "partial inhibitors".

(e). O'Neill et al. (1979b) have described a C4-deficient

patient with no plasma Ch and Rg antigens, but whose red cells type positive for both. Family members, typed C4*FS, have Ch antigens in plasma and on red cells, but Rg antigen only on their cells. The F product in all cases was reported to be non-functional in haemolytic overlay.

5. Similarities between C4 and other complement proteins

C4 shares structural and functional characteristics with other complement proteins:

a. C3, C4, and C5 are each activated by cleavage of a small peptide of about 80 residues from the N-terminus of its single (C5) or larger (C3) or largest (C4) peptide chain. C3a, C4a, and C5a have been sequenced and are homologous (see Hugli, 1981). In addition, C4a has been shown to possess biological activity similar to C3a and C5a.

b. C3 and C4, in common with the plasma protease inhibitor α_2-macroglobulin, each possesses an internal thiolester bond which is broken during activation of the molecule generating a reactive acyl group which is involved with the covalent binding reaction to target surfaces (Tack et al., 1980). These thiolester links occupy homologous positions in the molecules and are contained in areas of near identical amino acid sequences in the three molecules (Campbell et al., 1981).

c. Both C3 and C4 are produced as single-chain precursors.

d. Active C3 and C4, and also active α_2-macroglobulin each undergoes comparable denaturant-induced autolysis (Sim and Sim, 1981) which involves reactive sites presumed to be close to or the same as those involved in covalent binding reactions.

e. The structural genes for the complement components, C4, Factor B, and C2 are closely linked and situated within the major histocompatibility complex on chromosome six. This gene cluster will be discussed in the next section.

C. Complement alleles and the major histocompatibility complex

The genetic information which codes for the major histocompatibility complex in man is located on the short arm of chromosome six. Included in this cluster are genes for the leukocyte antigens, HLA-A, -B, -C, -D, and -DR, structural genes for complement Factor B, C2 and C4, and putative immune response and disease susceptibility genes. The gene for 21-hydroxylase has also been localized to this region as has the locus for the red cell enzyme glyoxalase I. A schematic representation of the MHC region is shown in Figure I-6.

1. Histocompatibility antigens

The HLA system includes five loci, with the A-locus

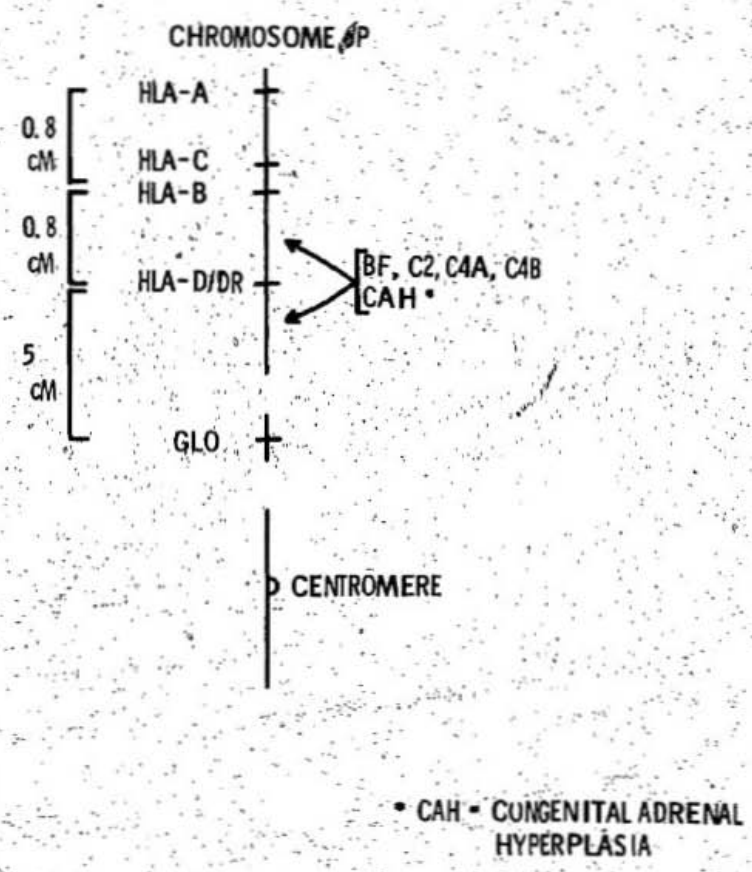


Figure I-6: Loci mapped to the short arm of chromosome 6 (after Fleischnick et al., 1983). Distances between loci are given in centimorgans (cM).

being most distal and the D/DR loci most proximal to the centromere. The positions and map distance of these loci have been shown by studies of families with chromosomal rearrangements (Lamm et al., 1974, Pearson et al., 1979), of human-hamster cell hybrids (van Sommeren et al., 1974), of human-mouse cell hybrids (Jones et al., 1976), and by reports from various laboratories of recombinant families.

The A, B, and C antigens are membrane glycoproteins, each consisting of one light chain, β_2-microglobulin, and one heavy chain. It is the heavy chain which carries the determinants recognized by HLA-typing sera and which is encoded in the MHC region. Although the antigens are widely distributed on most nucleated cells, typing is usually performed on peripheral blood lymphocytes.

Products of the D-locus are less well characterized. They are lymphocyte-activating determinants measured by the proliferative response of peripheral blood lymphocytes in mixed lymphocyte culture. Very little is known about the structure of D-determinants. They are known to be present on B-lymphocytes and cells of the monocyte-macrophage series, and to be associated with, but not identical to, the serologically-defined B-cell, or HLA-DR, alloantigens.

HLA-DR, or D-related, antigens are also membrane glycoproteins, known to occur on B-lymphocytes, monocytes, sperm cells, and stimulated T-cells. Each DR molecule consists of two polypeptide chains, α and β, and it is the

β-chain which carries the polymorphic determinants identified serologically in B-cell enriched peripheral blood lymphocyte preparations. A third invariant polypeptide chain has been detected (Jones et al., 1979).

The discovery of DR-related specificities has uncovered further complexity in this region. There is mounting evidence for the existence of additional loci linked to DR and coding for one or both chains of at least two DR-related molecules, identified as the DC and SB series of antigens.

2. Linkage disequilibria between alleles of the HLA-loci

The most remarkable feature of the HLA system is the extreme degree of genetic polymorphism. More than 90 alleles have been recognized at these loci. There is considerable variation in gene frequencies from population to population and from race to race. As these alleles are encoded by linked genes, they are inherited as haplotypes. Known alleles at the serologically-defined loci make possible some 150,000 haplotypic combinations.

Certain antigens are non-randomly associated or are in linkage disequilibrium, occurring together on a haplotype more frequently than would be expected from their individual frequencies. Some positively associated pairs of alleles are given in Table I-4. The various combinations of alleles in linkage disequilibrium vary in frequency

Table I-4: Some linkage disequilibria and haplotype frequencies of pairs of HLA-A, -B, -C, and -DR antigens in European Causcasoids.(1)

Haplotype		Δ x 100 (2)	HF x 100 (3)
A1	B8	57.2	20.6
Aw23	B12	17.6	19.3
A29	B12	27.3	33.1
Aw30	B18	16.6	17.0
Aw33	B14	6.4	6.6
B12	Cw5	40.2	48.2
B15	Cw3	28.3	29.9
B17	Cw6	35.4	38.7
B18	Cw5	19.4	23.6
Bw22	Cw1	11.4	12.2
Bw22	Cw3	18.9	20.7
B27	Cw2	22.6	22.7
Bw35	Cw4	84.2	89.7
B40	Cw3	24.5	26.4
B7	DR2	37.2	46.2
B8	DR3	62.3	70.1
B12	DR7	26.7	41.3
B13	DR7	16.8	18.5
B17	DR7	22.8	29.3
B18	DR3	12.9	18.2
Bw35	DR1	15.1	19.2

(1) Adapted from Bodmer and Bodmer, 1978 and van Rood et al, 1981.
(2) Δ = observed haplotype frequency - expected haplotype frequency.
(3) HF = haplotype frequency.

between populations. The phenomenon may be explained in a variety of ways: by the occurrence of a new mutation which has not had time to be distributed randomly by translocation throughout the various haplotypes; by founder effects and bottlenecks in particular populations; by recent admixture of two equilibrium populations; by selective pressure maintaining preferred combinations.

3. Chromosomal position of the complement loci

MHC recombinant data suggest that the complement loci lie between HLA-B and HLA-DR. A large number of A-B recombinants informative for BF have been described (by, for example, Teisberg et al., 1975; Bijnen et al., 1976; Olaisen et al., 1981). In all cases BF segregates with HLA-B. Olaisen et al. (1981) have reported four A-B crossovers where an informative C4 remains linked to HLA-B. Raum et al. (1981) have described two A-B recombinants, one informative for C4 where C4 remains linked to B and one informative for BF, C4 and C2 in which the alleles for all three complement components segregate with HLA-B. Dewald and Rittner (1979) have reported a single A-C crossover where C4 segregates with HLA-C and B, separating from HLA-A.

At least twenty HLA-B - HLA-DR recombinants informative for one or more of the complement loci have been observed. In eleven cases, complement alleles segregated with HLA-B (see, for example, Hawkins et al., 1980, for BF,

C2; Bruun-Petersen et al., 1981, for BF, C4; Olaisen et al., 1981, for BF, C4, C2; Rittner et al., 1976, for BF). In nine cases, complement alleles segregated with HLA-D/DR (Hawkins et al., 1980, for BF; Rittner et al., 1980b, for BF and C4; Suciu-Foca et al., 1980, for BF; Raum et al., 1981, for C4, BF, C2; Bruun-Petersen et al., 1981, for C4; Olaisen et al., 1981, for BF).

No recombinants have been observed among BF, C2, and C4, suggesting that the loci are very close together. Little is known about their relative order. Awdeh et al. (1981a) have reported strong linkage disequilibrium between the C4 haplotype A4B2 and the C2-deficiency allele. The occurrence of 24 A4B2 and a single A4BQ0 on 25 C2Q0 haplotypes suggests that the C4A-locus is closer than C4B to the locus for C2.

4. Linkage disequilibria between complement alleles and other alleles of the MHC

Table I-5 lists associations for Caucasoids which have been found in two or more separate studies. In addition, polymorphisms of BF, C4, and C2 have been described in Japanese populations. Tokunaga et al. (1981) have reported frequencies of approximately 94% C2*C, 3.4% C2*A, 2.2% C2*B, and 0.6% C2*A^T. C2*A is positively associated with HLA-B15, while C2*B is positively associated with HLA-Bw61. Horai et al. (1979) have found non-random associations between BF*S and HLA-B7, BF*F and HLA-B12, BF*F and

Table I-5: Associations between complement alleles and other alleles of the major histocompatibility complex, found in two or more studies.

Complement allele	MHC alleles
C2*Q0	A10(25) B18 Dw2/DR2, C4*A4B2, BF*S
C2*2	C4*A4B2
BF*S	B7, B7 Dw2, B8, B40, C4*S
BF*F	B12, B35, B15, B37
BF*S1	B21(50), B21 DR7 or 3, B12, B27, B13, B14
BF*F1	Cw5 B18 DR3
C4* (AQOB1)	B8, BF*S
C4*F (A3BQO)	B12, B35, BF*F
C4*F1 (A6B1)	B17, DR7
C4*M (AQOB2)	B40

HLA-B35. A report by Tokunaga et al. (1979) on C4 polymorphism gives associations between C4*S and HLA-A3, C4*F and HLA-Bw52, -B12 and -Bw54. Whether these C4 variants are comparable to those which have been reported for Caucasoids is unclear, however, since the investigators observed variants which they interpreted as combinations of two common alleles at a single C4 locus.

5. Immune response and /or disease susceptibility genes

Specific immune response genes linked to the murine H-2 region have been shown to control a variety of traits, such as susceptibility to infection by Gross leukemia virus (Lilly et al., 1964). This immune responsiveness appears to involve interaction between genes of loci in different I regions of the H-2 complex. The existence of these genes and the apparent homology between murine H-2 and the HLA-region have provided the rationale for investigating the relationship between the possession of particular MHC antigens and human diseases. The strongest correlation found was that for ankylosing spondylitis, where HLA-B27 was present in about 90% of propositi (Brewerton et al., 1973).

Table I-6 shows some of the diseases which have been reported as non-randomly associated with HLA alleles. Only the strongest associations are given. Juvenile insulin-dependent diabetes, for example, is shown here as associated with DR3/DR4. C4*B4, C4*B2 (Bertrams et al.,

Table I-6 : Some examples of HLA-associated diseases.(1)

Disease	HLA association
Ankylosing spondylitis	B27
Reiter's disease	B27
Acute anterior uveitis	B27
Rheumatoid arthritis	Dw4 DR4
Membranous glomerulonephritis	DR3
Coeliac disease	Dw3 DR3
Chronic active hepatitis	Dw3 DR3
Multiple sclerosis	Dw2 DR2
Type I diabetes	Dw3 DR3
	Dw4 DR4
Graves' disease	Dw3 DR3
Addison's disease	Dw3 DR3
Myasthenia gravis	Dw3 DR3
Psoriasis	Cw6
Behcet's disease	B5
Haemachromatosis (idiopathic)	A3
21-hydroxylase deficiency	B47

(1) adapted from Cudworth and Wolf, 1981.

1981a), C4*foS (Awdeh et al., 1980b; Lamm et al., 1980), BF*F1 (Bertrams et al., 1979; Kirk et al., 1979; Raum et al., 1979b; Lamm et al., 1980), C2*2 (Kirk et al., 1980; Lamm et al., 1980), HLA-B18 and HLA-B8 (see Dausset and Svejgaard, 1977) have also been reported to be increased in insulin-dependent diabetes patients. Similarly, HLA-B7 is increased in multiple sclerosis (Jersild et al., 1973).

It is generally assumed that the strength of the disease association provides information about the chromosomal position of a particular disease susceptibility gene, it being most strongly associated with the MHC marker closest to it. Other associations are thought to reflect linkage disequilibrium between the marker in question and other MHC alleles of a particular haplotype.

D. Levels of serum C4

Kohler and Muller-Eberhard (1967), using the method of single radial immunodiffusion (SRID) of Mancini (1965), found the mean serum level of C4 to be 43.6 ± 11.8 mg/ml. Subsequent reports, using the same technique, have given mean values of 25 - 45 mg/ml but there is a considerable degree of individual variation and an acceptable normal range as great as 15 - 100 mg/ml. The techniques of electroimmunoassay, nephelometry, and fluorometry have also been used for C4 determinations, with nephelometry giving

higher values than SRID (Bruver and Salkie, 1975) and fluorometry lower (Koelle and Bartholemew, 1982).

1. Sex, race, age

No differences in serum C4 have been observed with respect to sex in either adults (Kohler and Muller-Eberhard, 1967) or in children (Roach et al., 1981), nor have racial differences been demonstrated.

Fireman et al.(1969) found that C4 levels increased proportionally with gestational age, and comparison of cord and maternal plasmas indicated that C4 levels of cord plasmas were approximately 50% of that found in maternal plasma. Newborn infants have been shown, however, to possess 70 - 100% of that found in normal adults (Strunk et al., 1979) and Gallery et al. (1981) have observed significantly elevated C4 levels in pregnant women.

Norman et al. (1975) showed that, although there was a significant relationship of C4 level to age throughout the 0-14 year age range, 90% of adult levels was attained by two years of age, and essentially adult levels by the age of six.

2. Disease

a. Acute phase

C4 is widely regarded as an acute phase protein, although the actual amount of empirical data is small.

There are no reports of post-surgical changes in C4 levels, only very small changes after myocardial infarction (see Killingsworth and Killingsworth, 1981) and moderate increases during serous meningoencephalitis, peritonsillitis, and influenza A (Ganrot, 1974).

b. Other diseases

Low levels of serum C4 have been reported for a small number of diseases. C4 can be decreased in systemic lupus erythematosus due to activation of the complement system by circulating immune complexes (Killingsworth and Killingsworth, 1981) and by the same mechanism in rheumatoid arthritis (Nitsche et al., 1981). Low mean levels have also been reported in insulin-dependent diabetes patients (Vergani et al., 1982), in some HLA DR2+/DR2+ multiple sclerosis patients (Nerl et al., 1980), and in HLA-B8+ Graves' disease patients (Tom and Farid, 1981). It seems likely, however, that low C4 levels in diabetes and Graves' disease result from possession of particular disease-associated C4 null alleles (see Discussion).

3. C4 deficiency

a. Complete deficiency

Complete deficiency of C4 has been described for at least 16 cases in 11 families. C4-deficiency is not associated with a particular MHC haplotype, but nine of the sixteen cases are homozygous, suggesting consanguinity

(Hauptmann, 1982a). Most deficient individuals have lupus or lupus-like syndromes, which supports the suggestion (Lachmann and Hobart, 1978b) that very low levels of classical complement components predispose to the development of immune complex diseases such as SLE.

Two C4-deficient patients have been shown to be Rg-Ch- (O'Neill et al., 1978, Awdeh et al., 1981b), suggesting that the deficiency results from null alleles at the C4 structural loci. O'Neill et al. (1979b, 1980b) have described a family (see Section B4c, this chapter) in which the deficiency pattern is more complex. One C4-deficient individual had Ch and Rg antigens expressed on red cells, as measured by direct erythrocyte typing, but neither Ch nor Rg antigens in plasma. Other family members carrying the deficiency haplotype also had Rg antigen present on cells but absent from plasma. The investigators suggest that, in this family at least, there exists a mechanism for controlling Rg and Ch expression which is separate from that controlling the serum level of C4 protein.

b. Partial deficiency

The currently accepted model of C4 genetics assumes null alleles at each of the A and B loci with frequencies of approximately 0.15 for AQO and 0.10 for BQO (see Section B4b, this chapter). Demonstration of a C4-gene dosage effect has, however, been difficult. Awdeh et al.(1979) reported that the mean plasma C4 concentration (expressed

as % of normal) were 75 ± 24%, 70 ± 24%, and 67 ± 24% for for F/F, S/S, and F/S individuals as compared to 109 ± 44%, 101 ± 48%, and 100 ± 56% for FS/FS, F/FS, and S/FS individuals respectively. Thus, while 2-gene individuals had lower mean C4, there was considerable overlap between these and the 3- and 4-gene groups and individual variation was high. Olaisen et al. (1980) also compared C4 levels of 2-, 3-, and 4-gene individuals. They were able to show that a group of 2-gene individuals had significantly lower mean C4 but could show no significant difference between 3- and 4-gene groups. From these data, it seems likely that simple gene dosage effects only partially account for the very wide range of serum C4 levels observed.

E. Summary

The complement system is a series of proteins which, when activated, brings about lysis of cellular antigen and a variety of other biological effects such as anaphylatoxic activity, chemotaxis, opsonization, neutralization of viruses, and solubilization of immune complexes. Most of the complement proteins show genetic variation in the form of deficiency or of polymorphism.

The fourth component is produced in the liver or by macrophages as a single-chain precursor but takes the form of a three-chain glycoprotein in plasma. This glycoprotein,

when activated by C1 of the classical sequence produces the anaphylatoxin, C4a, and C4b. C4b exhibits sites for attachment to target surfaces, for association with C2a thereby forming the classical C3-convertase, for attachment to the receptors of a variety of circulating cells, and for proteolytic degradation by the control proteins C3b-inactivator and C4 binding protein.

C4 is highly polymorphic and production is directed by genes at two closely-linked loci, namely the A-locus whose products carry the Rodgers antigen, and the B-locus whose products carry Chido. The antigenic determinants which distinguish C4A and C4B, and also those which distinguish Rodgers and Chido, have been localized to a fragment of the α-chain of the C4 molecule called C4d. There are approximately eight alleles at the A-locus and four at the B-locus, including, for both loci, null alleles which occur with estimated frequencies of approximately 10-15%.

The loci for C4 are situated in the major histocompatibility complex of man, very closely linked to the genes for Factor B and C2, and, presumably, between the loci for HLA-B and HLA-DR. Population studies indicate that, as is the case for other alleles of this complex, C4 alleles show non-random associations with each other, with other MHC alleles, and with certain diseases.

Levels of plasma C4 are highly variable and are likely influenced by a number of factors, including disease

status, and genetic considerations such as the number of genes an individual possesses.

CHAPTER II AIMS AND OBJECTIVES

This project originated with the general objective of developing a reliable technique for identifying polymorphic variants of the complement factor, C4, in order to augment HLA data being accumulated in this laboratory on an extensive collection of families primarily from Newfoundland and Labrador but also from other parts of Canada. Many of these families include individuals suffering from diseases such as multiple sclerosis and insulin dependent diabetes mellitus.

The particular aims of the studies reported in this thesis are:

A. To determine the nature and number of C4 allotypic variants in this family material.

B. To investigate the relationship between Rodgers and Chido antigens and C4 allotypes.

C. To examine the relationship between the relative amounts of C4*A and C4*B protein in individual serum samples and C4 genotypes, in order to assess the usefulness of semi-quantitative techniques in C4-typing.

D. To estimate frequencies of C4 haplotypes and C4 genes in

the family-based population material.

E. To examine the relationship between C4 haplotypes and the major histocompatibility complex (MHC), in particular to look for non-random associations and linkage disequilibria between C4 haplotypes and MHC alleles.

F. To investigate the distribution of various extended MHC haplotypes, and of alleles within haplotypes, with a view to localizing more precisely the position of the C4 loci.

G. To investigate the distribution of C4 haplotypes in disease families, in particular those with multiple sclerosis and insulin-dependent diabetes mellitus.

H. To examine the factors which contribute to variation in levels of serum C4, in particular the relationship between C4 genotype and serum C4 concentration.

CHAPTER III: MATERIALS AND METHODS

A. BLood samples

1. Collection

Blood was collected by venipuncture into Vacutainer tubes. All samples were separated within one hour and stored immediately at -70° C. Where samples were collected by other laboratories, they were shipped on CO_2 ice. Samples were collected as serum, heparin plasma, or EDTA plasma. Care was taken to maintain samples in the best possible condition. Whenever a sample was used, it was thawed quickly in a 37°C waterbath, kept over crushed ice while being used, and refrozen as soon as possible.

2. Sources of samples

a. Family material

(1). The FS series of samples came from 65 local (Newfoundland) families. Donors were either healthy volunteers or family members of patients referred to the Clinical Immunology Diagnostic Laboratory for HLA-typing, immunoglobulin or complement assays, and/or other diagnostic tests of immune dysfunction. These patients included prospective kidney or bone marrow recipients, and individuals with diabetes, urolithiasis and other kidney

disorders, lupus, immunodeficiency, and a variety of immune-related disorders.

(ii). The LA series of samples were from a large family with multiple cases of myotonic dystrophy from two communities in coastal Labrador (Larsen et al., 1980).

(iii). The WC series of samples came from a large kindred and other families residing in three communities on the northwest coast of Newfoundland. These communities were extensively studied, both clinically and serologically, during the West Coast Health Surveys of 1974 and 1976 (Salimonu et al., 1980) and were shown, by pedigree analysis, to have a high degree of consanguinity. Twenty-one cases of lymphoreticular malignancy and embryonal tumour have been reported (Marshall et al., 1980b). Blood samples were collected in 1974 and 1976. A small number were collected in 1979 as part of the contribution of this laboratory to the VIIIth International Histocompatibility Workshop.

(iv). The WP series of samples came from 31 families provided by Dr. M. Schroeder. These were collected in the Winnipeg area mainly from immunodeficiency patients and their families.

(v). The MV series of samples were collected from nine families of Red Cross Blood Donors in Toronto by Dr. H. Mervart and selected primarily on the basis of their possessing unusual HLA-A, -B, and -C antigens.

(vi) The MS series of samples came from 60 families with

two or more cases of multiple sclerosis. Samples were collected by Dr. G. C. Ebers from families of patients at the MS Clinic at University Hospital, London, Ontario (Ebers et al., 1982).

b. Control panel

Fourteen healthy, unrelated members of the laboratory staff served as a control panel for C4 quantitative determinations. These individuals were bled weekly for six weeks and monthly for the following four months.

B. Complement factor 4

1. Immunofixation electrophoresis

Two variations of the method of Alper (1969) have been used in this study, and the details of these are summarized in Table III-1. Technique A is the method used by Teisberg et al. (1977) for C4-typing untreated heparin plasma samples. Samples were applied to a gel containing tris/citrate/borate buffer and run for four hours at 50v/cm.

Technique B is essentially the method developed by Awdeh and Alper (1980a) for typing the C4 variants of desialated plasma samples. Serum samples were pretreated with EDTA and neuraminidase and applied to a gel containing agarose, tris-glycine-gel buffer, and EDTA and run for

Table III-1: Two methods for typing C4 variants by immunofixation electrophoresis.

	Technique A(1)	Technique B(2)
Sample	EDTA plasma or heparin plasma	Serum with 0.02M Na_2 EDTA added
Pretreatment of sample	None	1 part neuraminidase (Sigma, Type VII, 50u/ml in potassium phosphate buffer) to 8 parts EDTA-serum incubated at room temperature 18 hours
Gel buffer	0.025M tris/citrate/ borate (Mauff,1978)	Vessel buffer, diluted 1:4
Gel	1% agarose (Litex) in 40 ml gel buffer	0.5% low EEO agarose (Seakem) in 50 ml gel buffer to which 0.005M Na_2 EDTA had been added
Plate size	20cm x 20cm	20cm x 25cm
Sample volume	7µl	5ul
Slot size	0.5cm	0.5cm
Cooling	Water-cooled metal support plate	Water-cooled metal support plate
Vessel buffer	0.05M tris/citrate/ borate, reused three times. 1000ml/tank	Tris-glycine (O'Neill, 1978) used once. 1200ml/tank
Wicks	Whatman #3 paper, (chromatography) 3 sheets	Whatman #3 paper, (chromatography) 2 sheets

continued

Table III-1: continued.

	Technique A(1)	Technique B(2)
Run	4 hours, 50V/cm (800V, 50mA)	3 hours, 550-560V (80mA)
Overlay	0.7 ml anti-C4 undiluted, applied directly to the gel	0.4 ml anti-C4 diluted 1:3 with PBS, applied directly to the gel
Incubation	1.5 hours, 37°C	1 hour, 37°C
Wash	24 hours in two changes of PBS, 24 hours in water	Press 10 minutes 18 hours in PBS, press 10 minutes 1 hour in water
Stain	0.1% Coomassie blue	0.2% Coomassie blue

(1) From Teisberg et al. (1977) for typing untreated samples.
(2) From Awdeh and Alper (1980a) for typing neuraminidase-treated samples.

three hours at approximately 30v/cm.

The electrophoresis tank used for both methods contained a metal support plate which was cooled by a continuously running stream of tap-water (at 2–10°C). Paper wicks were used to connect gel to vessel buffer and a glass plate served as condensation lid.

For routine C4-typing the overlay was goat anti-C4 (Atlantic Antibodies) applied directly to the gel after electrophoresis. Control plates were repeated using rabbit anti-C4 (Behring) and rabbit anti-C4 (Pel-freeze).

2. Electrophoresis with haemolytic overlay.

After electrophoresis with desialated samples by Technique B, Table III-1, the gel was overlaid with a mixture of agarose, sensitized sheep erythrocytes, and C4-deficient guinea pig serum, prepared in the following manner:

15 ml of molten 0.75% indubiose (IBF) in complement fixation diluent (Oxoid), 0.5 ml 10% sensitized sheep erythrocytes (heat-inactivated rabbit anti-sheep ambo-ceptor, Grand Island), and 0.35 ml C4-deficient guinea pig serum were mixed at 45°C and poured on a 13cm x 20cm sheet of Gelbond film (FMC), using gasket, clips, and a level surface. This gel was allowed to set, applied face down to the gel containing separated samples, and incubated at 37° C. Bands of haemolysis appeared in 10–30 minutes. The

overlay was removed and fixed in 1% glutaraldehyde for 20 minutes.

3. Crossed immunoelectrophoresis (CIE).

The method of Laurell (1965) was used with the following modifications:

a. First run

Two gels were prepared, each containing 32 ml of 0.5% low EEO agarose (Seakem) in gel buffer (technique B, Table III-1) poured on a 9cm x 18cm strip of Gelbond. Desialated plasma samples (5 µl) were applied to eight 7-mm slots made 1cm apart, 2cm from one long edge of each gel as shown in Figure III-1a. Both gels were electrophoresed three hours on two independent sets of electrophoresis equipment.

b. Second run

A mixture of 40ml of 0.5% low EEO agarose and 0.3ml of anti-C4 in gel buffer (technique B, Table III-1) was poured on a 20cm x 25cm glass plate at 40° C and the gel allowed to set. Both gels from A were carefully applied face down and side by side on this plate, as shown in Figure III-1b. This plate was electrophoresed 2.5 hours at 500V (95mA to start). The Gelbond overlays were removed, washed, and stained as in Technique B, Table III-1.

To quantify A and B variants, A and B CIE curves were cut from the stained, dried Gelbond films and weighed.

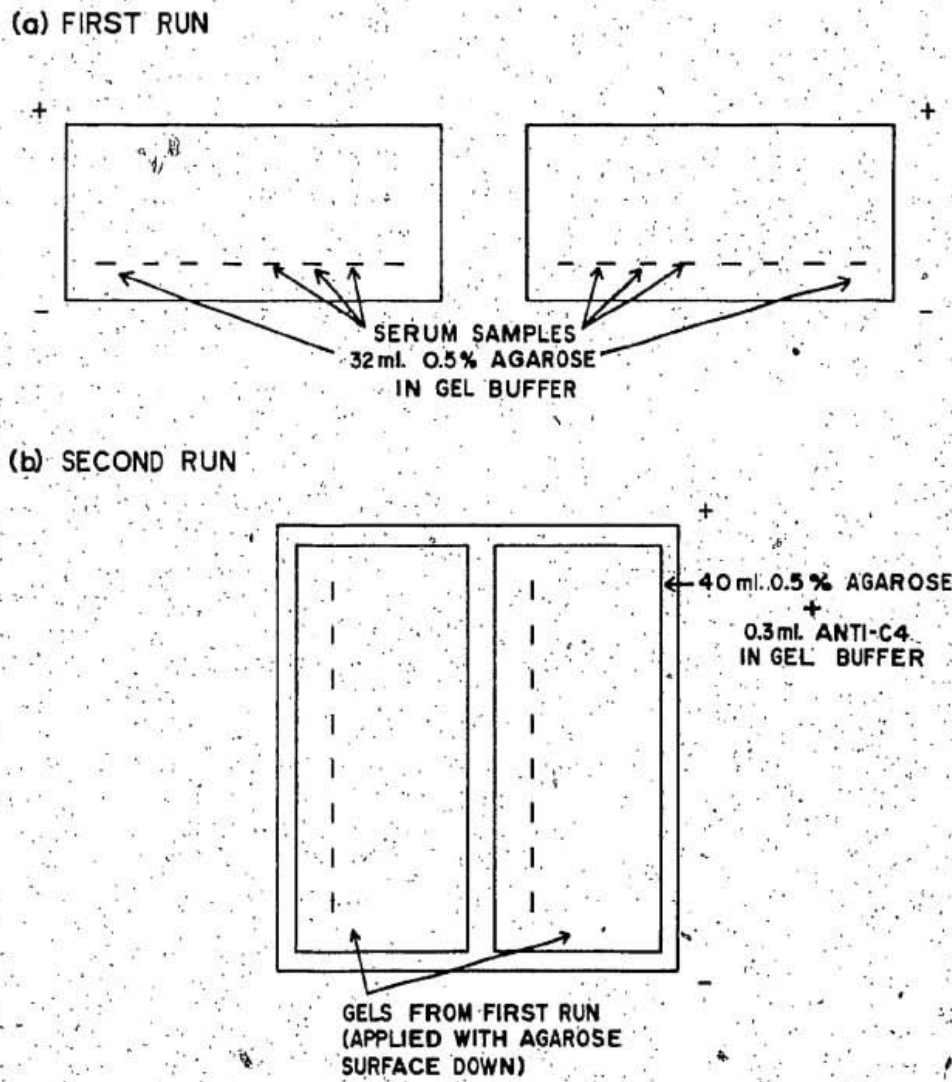


Figure III-1: Gels for crossed immunoelectrophoresis of C4.

4. Rodgers and Chido antigens.

Rodgers typing was performed in tubes using serial dilutions of anti-Rg antibody (provided by C.Giles, Hammersmith Hospital, London and by R. Berger, Toronto). To two volumes of antibody was added one volume of test serum. After five minutes, one volume of a 1.5% suspension of Rg positive cells (group O) was added. The tubes were incubated at 37° C for 45 minutes, the red cells washed three times, and one drop of anti-human IgG (Ortho diagnostics) was added. The tubes were centrifuged, the cells poured onto a microscope slide, and the agglutination read under a microscope. A control (test serum + cells + anti-human IgG) was included for each serum tested. A serum was judged to be Rg+, Rg-, or Rg(partial inhibitor) depending on the inhibition pattern obtained:

Anti-Rg diluted	1:64	1:128	1:512	Rg type
Test serum I	-	-	-	Rg+
II	+++	+++	++	Rg-
III	++	+	+-	Rg partial

Chido typing was performed in the same way. Anti-Ch (from C. Giles and R. Berger) was diluted 1:32, 1:64, and 1:256. A serum was considered to be Ch+ if it fully inhibited agglutination and Ch- if it did not inhibit. No serum was found that could clearly be classified as

Ch(partial inhibitor) although a small number of samples could not be definitely typed.

5. Protocol for C4-typing the samples

a. Samples were run as families (for FS, LA, WC, and MV samples) or in random order (WP and MS samples).

b. Each phenotype was recorded for each sample as it was read from the plate.

c. For each sample possessing both A and B variants, a qualitative impression of the relative concentration was noted, for example, A heavy, B weak.

d. Phenotypes for each family were entered on its pedigree and C4 genotypes were assigned as completely as possible. For the MS samples only, samples which were phenotyped A3B1 were subjected to crossed immunoelectrophoresis when A3B1 was the only phenotype in the family.

e. Entry or founder haplotypes were deduced for each family and stored in a computerized haplotype file (Datatrieve).

6. Analysis of C4 family material

C4 phenotype frequencies were determined by direct counting of all samples typed. Haplotype frequencies were determined by direct counting from a list of all entry haplotypes in the total family material. Entry haplotypes are founder or unrelated haplotypes selected from the family pedigrees. Gene frequencies were estimated directly

from entry haplotypes.

C. Other MHC antigens

1. Histocompatibility antigens

HLA-A, -B, -C, and -DR typing was performed by the two-stage lymphocytotoxicity assay by the investigators in whose laboratories the blood was collected. Newfoundland HLA typing was performed in the tissue typing laboratory supervised by Mr. J. M. Barnard.

A portion of the West Coast (WS) samples was HLA-typed in 1974 according to 1974 standards, so that some "splits", for example, of B12, were not identified. For purposes of association analyses these early categories were retained for all data, e.g. B12 contains all samples identified as B12, B44, or B45.

2. Complement Factor 2

Variants of the second component of complement were typed according to the method of Meo et al. (1977). Typing was performed in the laboratory of Dr. W. H. Marshall and the data made available to me. Serum proteins were separated by isoelectric focusing in the pH range 4 to 8 on thin-layer polyacrylamide slabs. C2 bands were developed by the zymogram method with functional haemolytic overlay

containing 5.2ml 1.5% indubiose in CFD, 0.35ml 10% sensitized erythrocytes (heat-inactivated rabbit anti-sheep amboceptor), and 0.5ml C2-deficient human serum.

Variants were identified as combinations of three allelic patterns, C2*1, C2*2, and C2*Q0. The designation C2-1* was used to describe the haplotype found in families displaying the phenotype C2-1 only, that is, for haplotypes from which the null allele, C2*Q0, could not be excluded.

3. Complement Factor B

Factor B typing was performed by the technique of immunofixation electrophoresis. This typing was performed in the laboratory of Dr. B. Larsen according to the method of Alper et al. (1969). Serum proteins were separated by zone electrophoresis in barbital buffer. BF bands were developed by overlaying the preparation with goat anti-Factor B (Atlantic Antibodies). Variants were identified as combinations of four allelic patterns, S, F, F1, and S1.

4. Analyses of extended haplotype data

Complete haplotypes were derived by applying HLA, C2, and BF data to pedigree sheets containing C4-haplotyped individuals.

Two-way associations between all pairs of HLA-B,

HLA-DR , BR, C2 alleles, and C4 haplotypes were determined by two methods:

(a) The chi square method

i. $X^2 = \frac{N(|ad - bc| - N/2)^2}{(a+b)(c+d)(a+c)(b+d)}$ (with Yates' correction)

ii. $p = \frac{(a+b)!(c+d)!(a+c)!(b+d)!}{N!a!b!c!d!}$ (Fisher's exact test)

where a,b,c,d are the cells of the appropriate 2 x 2 contingency table for the two alleles being compared, p is probability, and N is the total haplotypes tested for both antigens.

(b) Delta standard method

These calculations were suggested by Dausset et al. (1978) and Grange et al.(1981).

iii. Delta = D = Pab - Pa x Pb

iv. Standard delta = $Ds = \frac{D}{Dmax}$

v. For positive values of delta, D+:

D+max = Pa (1 - Pb) where Pa $\leq$ Pb, and

for negative values of delta, D-:

D-max = -Pa x Pb

where Pa and Pb are the observed frequencies of the

antigens (or alleles, or for C4, two-locus haplotypes) being compared, and Pab is the frequency with which they occur together.

Three-, four-, and five-way associations were determined by extending these formulas (Grange et al., 1981) so that

$$D = Pabc...z - Pa \times Pb \times Pc... \times Pz$$

$$D+s = \frac{D+}{Pa\,(1 - Pb \times Pc... \times Pz)}$$ where Pa $\leq$ Pb, Pc, ...Pz.

$$D-s = \frac{D-}{-Pa \times Pb \times Ps... \times Pz}$$

where Pa, Pb, Pc,...Pz are the frequencies of the alleles (or for C4, two-locus haplotypes) and Pabc...z is the observed frequency of the 3-, 4-, or 5-component haplotype.

D. Quantitation of C4 protein

Serum C4 concentrations were determined by the technique of single radial immunodiffusion (SRID, Mancini, 1965) using commercially prepared plates (Behring). The method was exactly as described by the manufacturer. Three dilutions of a known standard serum (Behring standard, 30

mg% C4) and 1:2 dilutions of the test sera were applied to the wells of the SRID plate in volumes of 5 µl. The same volume of a known control (Behring control plasma, 25mg% C4) was applied to every second plate. Two determinations were made for each sample.

Ring diameters were measured after 72 hours and C4 concentrations (mg%) were calculated from standard reference curves. Values for duplicate samples were averaged.

CHAPTER IV: RESULTS

A. Description and analyses of C4 electrophoretic patterns

1. Untreated samples

a. Phenotypic patterns after immunofixation electrophoresis

Samples from 520 individuals were C4-typed by technique A (Methods, Table III-1) with three common and six rare precipitin patterns being detected. A selection of these patterns is shown in Figure IV-1. The common phenotypes consisted of F, with 3-4 anodal bands, S with 3-4 cathodal bands, and FS with all 6-8 bands of F and S. The rare phenotypes consisted of combinations of F or S or both with the rare patterns D, I, M, or Sx. D and I were considered to be allelic to F. D resembled F but all bands were shifted slightly toward the anode. The bands of I were shifted slightly from F to the cathode. M was considered to be allelic to S. It resembled S but all bands were shifted toward the anode. Sx was identical to F but differed in Chido typing.

b. Genotypes

Genotypes were assigned by applying the phenotype information to family data, assuming two loci for C4 per chromosome. The occurrence of two null alleles, one at each

PHENOTYPES	FS	F	IS	DS	FMS	M or MS	S	FS	FS	FS
GENOTYPES	$\frac{FS}{FS}$	$\frac{Fso}{Fso}$	$\frac{Iso}{foS}$	$\frac{DS}{foS}$	$\frac{FS}{foM}$ or $\frac{fso}{foM}$	$\frac{foM}{foM}$ or $\frac{foM}{foS}$	$\frac{foS}{foS}$	$\frac{Fso}{FS}$	$\frac{foS}{FS}$	$\frac{FS}{FS}$

Figure IV-1: Precipitin patterns seen after immunofixation electrophoresis of heparinized plasma samples. Interpretations of phenotypes and genotypes are given below the photograph.

locus on the same chromosome, was assumed to be extremely rare, while a null allele at either locus of the same chromosome was assumed common. Thus individuals with the F pattern were genotyped Fso/Fso. Similarly individuals with the S pattern were genotyped foS/foS. FS phenotypes could be any one of four genotypes. A sample was typed FS/Fso if precipitation in the F region was much denser than S, and FS/foS if the S region was denser. FS patterns with F and S regions of roughly equal intensity could be genotyped only in those families where the F and S segregated clearly in other family members. Three families are shown in Figure IV-2. Of the 113 unrelated people in this sample, only 93 could be definitely genotyped. The proportions of C4 genotypes in these are shown in Table IV-1.

c. Haplotypes

There were 241 entry haplotypes in this material, meaning those in parents or derived from other founders in each family. Some of the haplotypes were drawn, not from the founder who was untyped, but from the offspring, which accounts for the discrepancy between number of unrelated people (113) and number of entry haplotypes. The distribution of C4 haplotypes is given in Table IV-2.

d. Chido and Rodgers typing

All individuals who carried the F pattern only were found to be Ch-Rg+ and those with S or M only were Ch+Rg-.

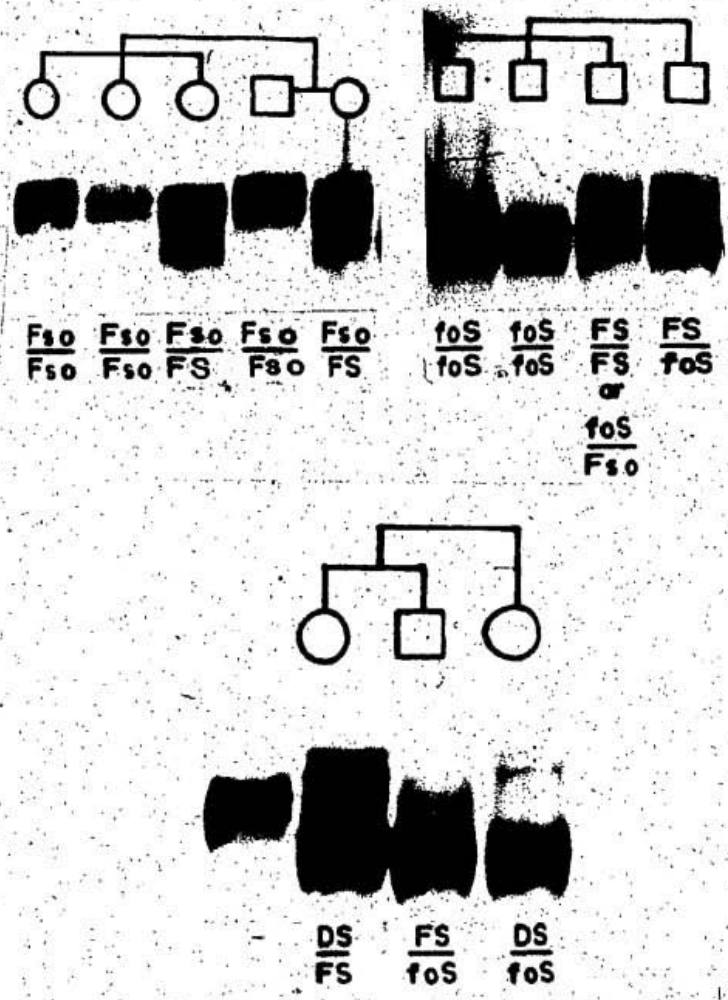


Figure IV-2: C4 genotypes of three families typed by immunofixation electrophoresis of heparinized plasma samples.

Table IV-1: C4 genotypes of 93 unrelated individuals using untreated plasma samples and technique A for typing C4 variants.

Genotype	Number observed
Fso/Fso	6
foS/foS	7
FS/FS	18
Fso/foS	11
FS/foS	21
Fso/FS	19
FS/DS	5
DS/foS	2
Fso/foM	1
Iso/foS	1
Fso/FM	1
Fso/FSx or Fso/foSx	1
Total	93

Table IV-2: Numbers and frequencies of entry (unrelated) haplotypes observed after C4 typing 520 untreated plasma samples from family material.

	Fso	FS	foS	DS	foM	FM	Iso	FSx	Total
No. observed	57	108	63	7	3	1	1	1	241
Frequency	0.237	0.448	0.261	0.029	0.012	0.004	0.004	0.004	1.00

Individuals with FS, FM, IS, and DS were Ch+Rg+. The Sx variant gave precipitin bands in the F region but was found on a Ch+Rg+ (FSx) haplotype. It was therefore an unusual Ch+ F or a very fast-moving S.

e. Typing with functional haemolytic overlay

When samples positive for the D variant were typed using functional haemolytic overlay, no sample tested had bands of haemolysis in the D region. Thus it is evident that this D gene product appears to be a non-functional variant.

2. Neuraminidase-treated samples

The letters A and B are used here to name variants observed by immunofixation electrophoresis of desialated samples in order to distinguish patterns obtained by this method from those seen using native C4, and to facilitate comparison with other investigators using this method.

a. Phenotypic patterns after immunofixation electrophoresis

All samples typed by technique A were retyped after neuraminidase treatment (technique B, Table III-1) and an additional 1337 samples were typed. Typical patterns obtained by this method consist of six to nine immunoprecipitin bands spread over a distance of approximately 2-3cm on the gel. The pattern most frequently seen has three anodal A bands and three cathodal B bands (Figure IV-3,

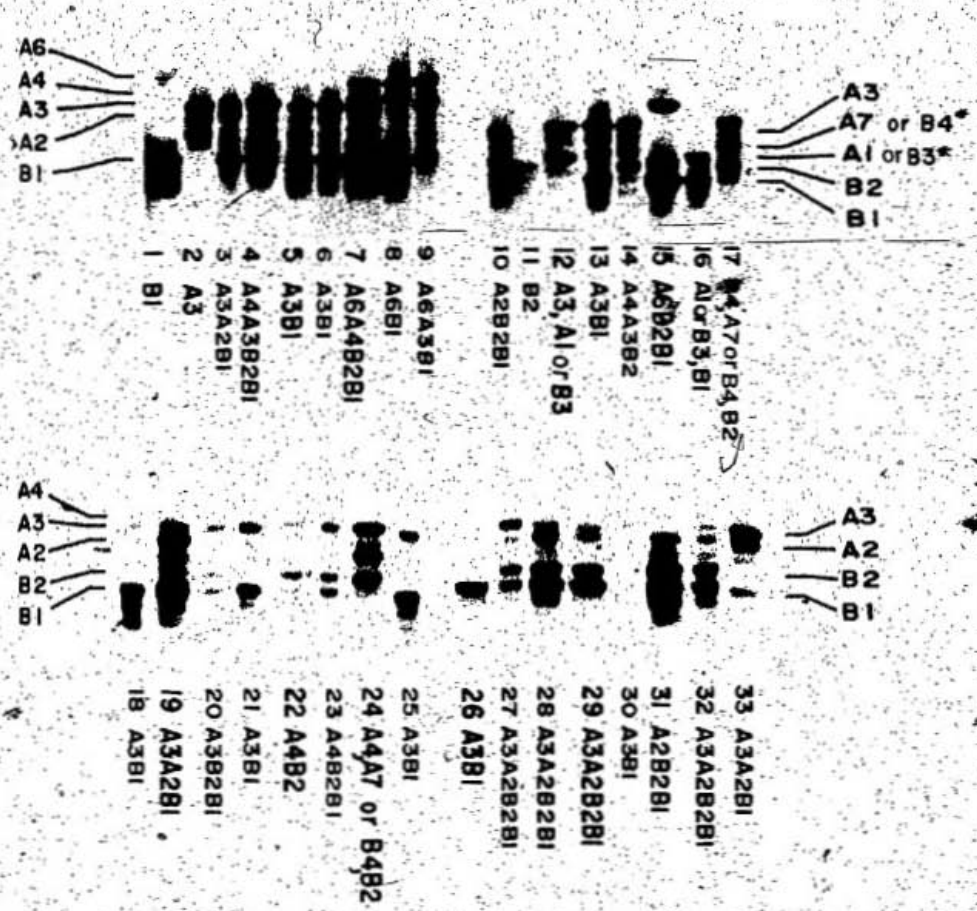


Figure IV-3: Some of the C4 phenotypic patterns seen after immunofixation electrophoresis of desialized plasma samples. Phenotypes are given directly below the photographs, and suggested gene products to the left and right of the photographs. C4A products are allelic as are C4B products.

* A and B patterns cannot be distinguished

sample #5).

Forty different phenotypic patterns were distinguished in the 1857 samples tested in this study. A selection of these is given in Figure IV-3. Table IV-3 summarizes the distribution of phenotypes obtained from all the individuals in all the families used. The phenotype A3B1 occurs in approximately 54% of the people tested. This pattern was used as a reference on all plates. Eighty-six per cent of the samples account for 12 phenotypes. The remaining 28 phenotypes are rare, some occurring in a single family only.

Some phenotypic patterns were difficult to interpret for the following reasons:

(i). A4 is displaced a small distance anodal to A3. A4A3 combinations (Figure IV-3, #4) are not double-banded. Instead, a single heavy first band covering the A4A3 region is usually seen, which, in some runs, is difficult to distinguish from A4 (Figure IV-3, #17, #22, #23, #24).

(ii). The A1 or B3 (#12) and A7 or B4 (#17, #24) patterns, when combined with others, give complex banding patterns which are occasionally blurred.

(iii). Samples with A3 and A2 together are not always identical. Sometimes the combination gives a weak A3 first band (#32), sometimes the reverse (#27, #33), and sometimes both are relatively equal (#3, #28, #29).

(iv). A3B1 phenotypic patterns differ in the relative

Table IV-3: C4 phenotypes in 1857 samples from the family material, typed by technique B.

C4 Phenotype	FS	LA	MS	MV	WC	WP	Total	Frequency
A3B1	249	64	279	31	278	100	1001	0.5390
A3A2B1	21	23	38		32	25	139	0.0749
A4A3B2B1	27	4	33	2	14	9	89	0.0479
A3BQO	18		3	1	37	6	65	0.0350
A4B2B1	10	3	18	3	21	5	60	0.0323
AQOB1	14	1	13	1	24	1	54	0.0291
A6A3B1	34		13	2	1	2	52	0.0280
A3B2B1	9		14		5	6	34	0.0183
A3B3B1	15		7		6	2	30	0.0162
A4A3B4B1	4		6	5	8	5	28	0.0151
A3A2B2B1	3		17	1	2	3	26	0.0140
A4A3B2	7		7	1	5	3	23	0.0124
A2B2B1	2		8	2	2	4	18	0.0097
A4A2B2B1			10		5	2	17	0.0092
AQOB2B1	1	9	5		1		16	0.0086
A4B2	3		10		1	1	15	0.0081
A3A2BQO	5		4		2	3	14	0.0075
A3B3	2		1		8	3	14	0.0075
A6B1	3		4			1	8	0.0043
A2B1			5		3		8	0.0043
A4B4B1			2		6		8	0.0043
A3A2B2			6		2		8	0.0043
A4A3B4					6		6	0.0032
A6A4B2B1	3		1			2	6	0.0032
A3B2			3		1	1	5	0.0027
A4A3B3B2			3		1		4	0.0022
A4A2B2			3	1			4	0.0022
A3A2B3B1			2			1	3	0.0016
A3A2B3			2			1	3	0.0016
A4A2B4			2				2	0.0011
A4A3A2B4	2						2	0.0011
A4B4B2						2	2	0.0011
A4B3B2	1						1	0.0005
A6A2B1	1						1	0.0005
A6A2B2B1	1						1	0.0005
A4A2B4B1			1				1	0.0005
A6A3A2B1	1						1	0.0005
A2B2			1				1	0.0005
AQOB3B1						1	1	0.0005
AQOB2						1	1	0.0005
Not typable	43	9	2		23	8	85	0.0458
Total	479	113	523	50	494	198	1857	1.000

intensity of A and B regions. (Figure IV-4).

b. The gene products

The patterns described here appear to be combinations of allelic gene products of two loci. A-locus products are anodal and similar in position to A3. B-locus products are cathodal and similar to B1. This distinction is not perfect, however, since a few products, particularly A1 and B3, A7 and B4, have banding positions directly between A3 and B1. In these cases electrophoretic position cannot be used in assigning the letters A or B and other criteria must be used (see below).

The gene products are illustrated schematically in Figure IV-5. Each product usually shows three precipitin bands of which the first (anodal or upper) stains most heavily. In some runs, only the first band is seen.

i. Products of the A locus

A3 is most commonly seen. Sample #2 in Figure IV-3 shows A3 alone and sample #6 shows this variant combined with B1.

A4 is shown in Figure IV-3, sample #22. The first band lies anodal to the first A3 band by approximately one-half position (one-half the distance between first and second A3 bands).

A6 has three bands such that the second band of A6 is just anodal to the first band of A4. The first band of A3

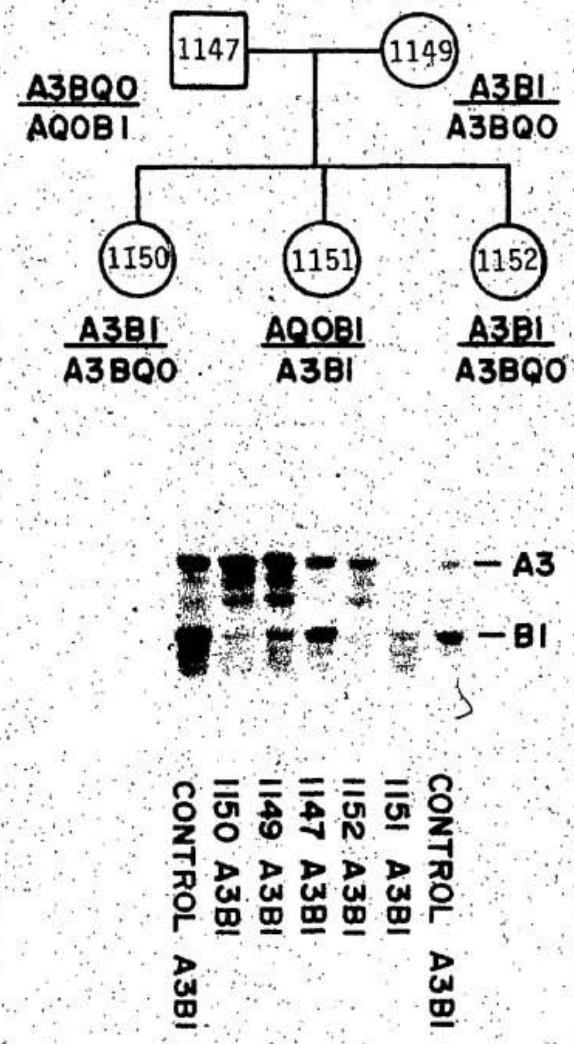
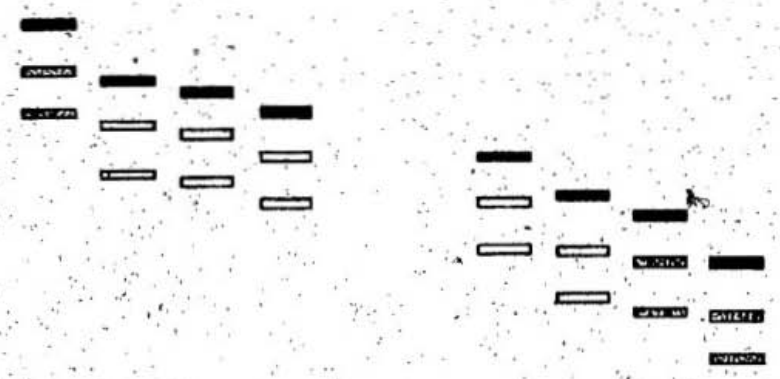


Figure IV-4: Different A3B1 patterns observed in a single family. Possible genotypes based on different staining intensities are given on the pedigree. Phenotypes are shown below the photograph.



A6 A4 A3 A2

B4 B3 B2 B1
or or
A7 A1

Figure IV-5: Products of the C4A and C4B loci seen after neuraminidase treatment. C4A products are generally Ch-Rg+, C4B products Ch+Rg-. B products show strong haemolytic activity after neuraminidase treatment.

lies directly between bands two and three of A6 (Figure IV-3, #7, #8, #9).

A2 is cathodal to A3, its first band lying just ahead of the second A3 band (Figure IV-3, #10, #31). The second and third bands of A2 are rarely distinguished clearly.

A7 is identical in position to B4.

A1 is identical in position to B3.

AQ0 is the designation for the null allele. Individuals with no observable A patterns are assumed to be homozygous AQ0/AQ0.

ii. Products of the B locus

B1 is the most common. It has three bands cathodal to A3. Sample #1 in Figure IV-3 shows B1 alone and it is combined with A3 in samples #3, #5, and #6.

B2 is one full position anodal to B1 so that the second band of B2 overlaps the first band of B1 (Figure IV-3, #11, #22, #28).

B3 is identical to A1. There are occasionally three bands with the first lying slightly cathodal to the third band of A3 (Figure IV-3, #12) but the first band is usually the only one clearly distinguished (#16).

B4 is identical to A7. The first band of this variant, usually the only one seen, is slightly anodal to the third band of A3, (Figure IV-3, #17, #24).

BQ0 is the symbol for the null allele and individuals with no B patterns are assumed to be BQ0/BQ0.

c. Genotypes

Genotypes were assigned using pedigree information and the two previously stated assumptions, that there are two loci per chromosome for C4, and that a null allele (AQ0, BQ0) is common at one locus or the other but very rare at both.

The four families in Figures IV-6 to IV-9 illustrate how segregation patterns were interpreted. Phenotypes are given underneath the samples. Genotypes are shown on the pedigrees. The letters a, b, c, d, etc. refer to entry or founder haplotypes derived from each family.

There were three situations where genotypes were difficult to assign. These are (i) families in which A3B1 was the only phenotype, (ii) families with the rare patterns A1 or B3, or A7 or B4, and (iii) families with either A3 and A2 or B1 and B2 together.

i. Families in which A3B1 was the only phenotype

Many families were not informative for either A3 or B1, that is, these genes products were not paired with other easily identifiable allelic products in any individual in the family. Assignment of genotypes in these individuals was difficult since their genotypes could be any one of five haplotypic combinations, A3B1/A3B1, AQ0B1/A3BQ0, A3BQ0/A3B1, AQ0B1/A3B1, and AQ0BQ0/A3B1. The last of these was not considered since AQ0BQ0 was assumed

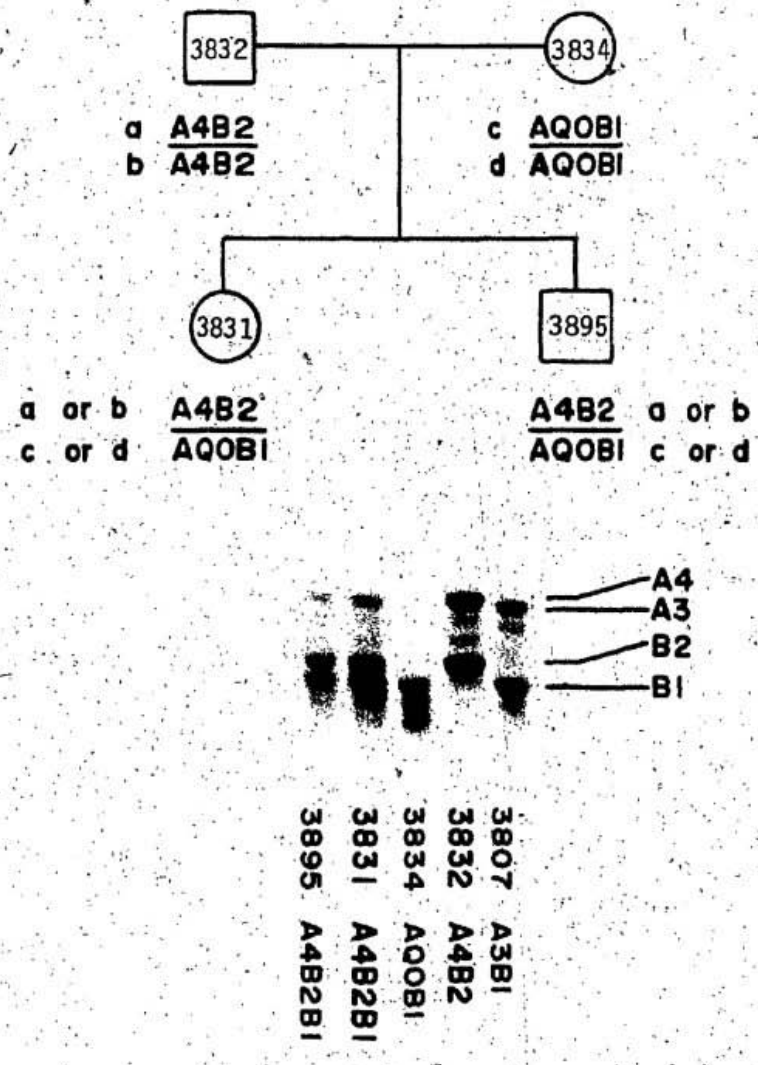


Figure IV-6: A family which illustrates segregation of the C4 haplotypes A4B2 and AQOB1. Entry or founder haplotypes are designated a, b, c, and d. Phenotypes are given beneath the photographs.

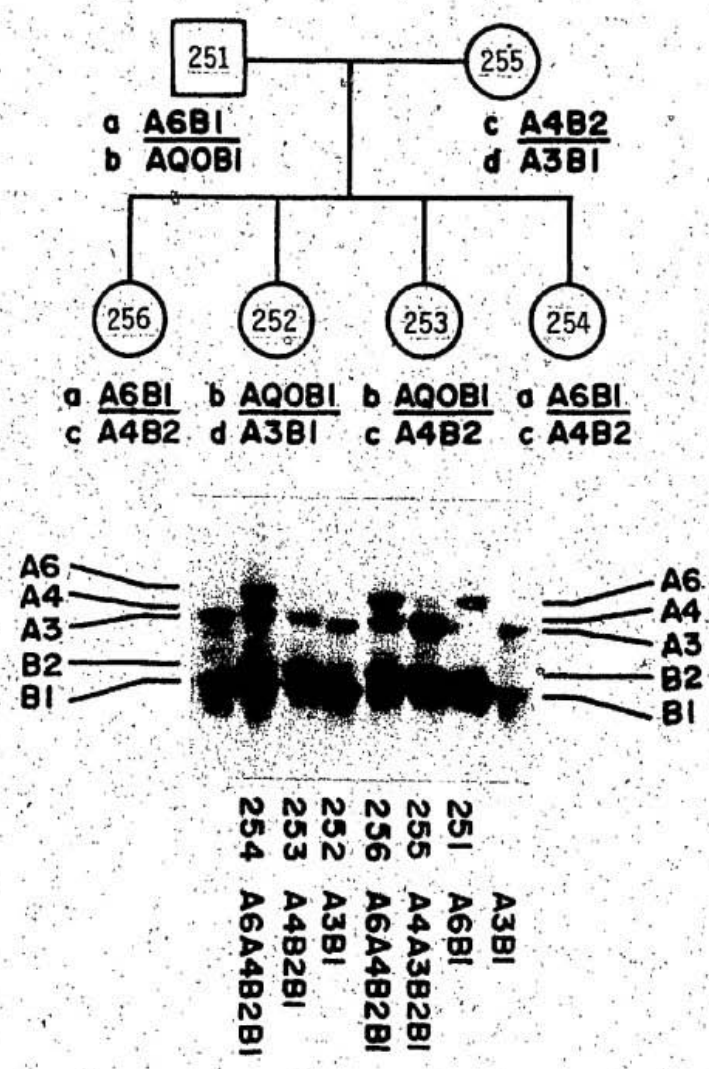
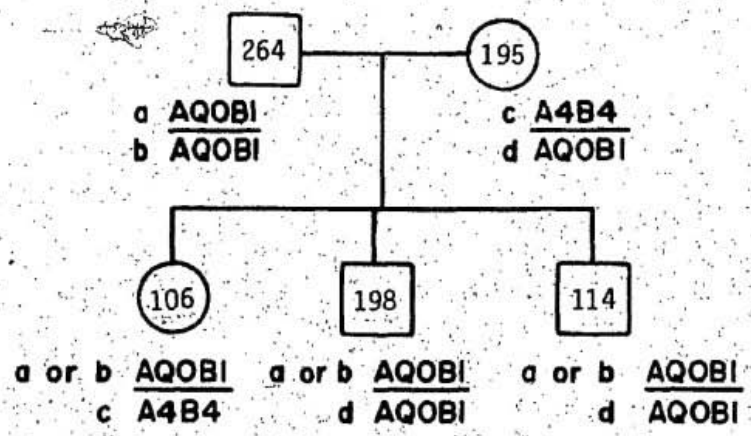


Figure IV-7: A family which illustrates segregation of the C4 haplotypes A6B1, AQOB1, A4B2, and A3B1. Entry haplotypes are designated a, b, c, and d. Phenotypes are given below the photograph.



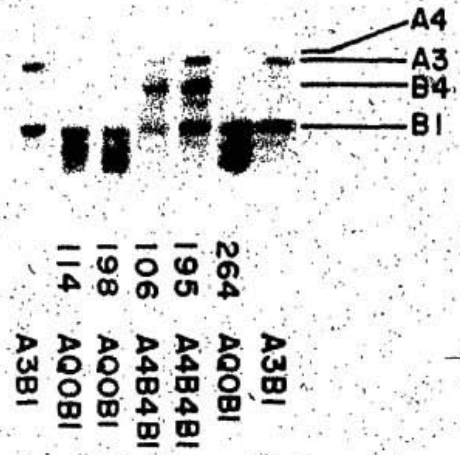


Figure IV-8: A family which illustrates segregation of the C4 haplotypes AQOB1 and A4B4. Entry haplotypes are indicated as a, b, c, and d. Phenotypes are given below the photograph.

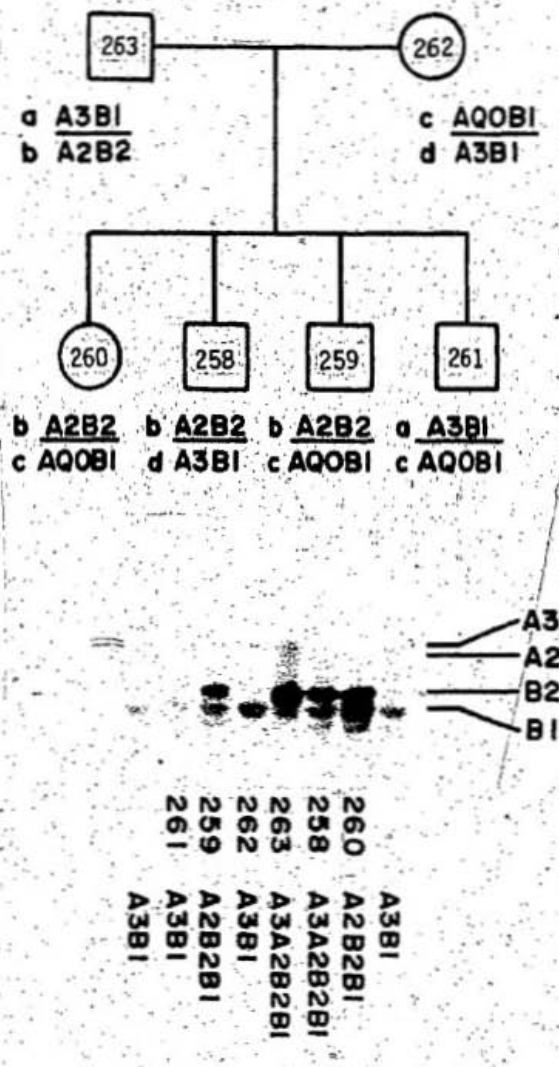


Figure IV-9: A family which illustrates segregation of the C4 haplotypes A3B1, A2B2, and AQOB1. Entry haplotypes are designated a, b, c, and d. Phenotypes are given below the photograph.

to be very rare.

Two criteria were considered in distinguishing AQOB1/A3B1 and A3BQO/A3B1 from A3BQO/AQOB1 and A3B1/A3B1, namely, the relative intensities of the A and B patterns on the immunofixation plate, and the results of crossed immunoelectrophoresis.

A qualitative impression of the relative concentration of A and B immunoprecipitins, as indicated by staining intensity on the immunofixed plate, was recorded with each phenotype. If the A and B components of the A3B1 pattern were equal, the genotype was initially judged to be A3B1/A3B1 or A3BQO/AQOB1; if A3 was heavier the genotype was judged to be A3BQO/A3B1; if B1 was heavier the pattern was judged AQOB1/A3B1. Individuals with known A3BQO/A3B1 and AQOB1/A3B1 usually showed these uneven staining patterns. Sometimes they did not. For this reason, and because A3B1/A3B1 and AQOB1/A3BQO could not be distinguished by any means, a "conservative" approach was eventually adopted in assigning genotypes. In families where the staining patterns were not supported by the existence of A3BQO or AQOB1 in informative combinations, the A3B1 phenotype was genotyped A3*B1*/A3*B1*. Where the alleles at one locus were clearly seen to segregate, but not the alleles at the other, individuals were typed A3*B1/A3*B1 or A3B1*/A3B1*, etc. In all cases, the asterisk means "or null".

Figure IV-4 shows such a family. There are no definite A3BQO or AQOB1 individuals. The pedigree above the figure shows the interpretation of each phenotype made by comparing intensities of the A and B patterns. All were eventually recorded as A3*B1*.

An attempt was made both to quantify these qualitative impressions of gene dose and to further investigate the assumption that the relative amounts of A and B immunoprecipitins in a sample indicates the number of A and B genes present. Samples were separated by crossed immunoelectrophoresis (CIE) on Gelbond film, and, in order to estimate the area under the A and B curves obtained, each curve was carefully excised and weighed.

If the relative size of the A and B curves obtained from a sample after CIE reflects the number of structural genes present, then one would expect to obtain, in a large sample, three clusters of individuals. One cluster would contain 3-gene individuals, AQOB1/A3B1, with A:B curve ratios of approximately 0.5. A second cluster would include the 2- and 4-gene individuals, AQOB1/A3BQO and A3B1/A3B1, with A:B curve ratios of approximately 1.0, and the third would contain 3-gene individuals, A3BQO/A3B1, with A:B curve ratios of approximately 2.0.

Ten control samples, phenotyped A3B1, were subjected to CIE, and replicated once on the same electrophoretic plate, and twice on a second plate. The mean A:B ratios for

the ten samples were 1.002 and 1.067 on plate 1, and 1.068 and 1.025 on plate 2. An analysis of variance was performed using the A:B ratio as the dependent variable against the independent variables sample (S), same plate replication (R), and different plate replication (P). The results are given in Table IV-4. The column on the right of this table gives the standard deviation between individuals (0.69) and within individuals (0.11). The latter figure can be taken as an estimate of the error of the method, which is approximately 10%. The between-individual variation is high, at 0.69 or approximately 66%, which is to be expected if the ten A3B1 samples included individuals with A:B ratios of 0.5, 1.0, and 2.0.

One hundred fifty-four samples from MS families showing the A3B1 phenotype only were subjected to CIE. Figure IV-10 shows the distribution of A:B curve ratios obtained. It can be seen that the range of ratios is from 0.15 to 3.0 with two very high ratios at 4.25 and 5.75. The 154 individuals do not all have the same or similar A:B curve ratios since, if this were a normal distribution with a mean of approximately 1.0 and standard deviation due to experimental error only, the values would range from 0.67 - 1.33 ($\pm$ 3 S.D. taken as 0.11). The between-subjects standard deviation for this distribution is 0.69 as in the controls. The distribution is therefore positively skewed and not symmetrical around a mean of 1.0.

Table IV-4: Analysis of variance of the A:B curve ratios obtained by crossed immunoelectrophoresis from 10 C4*A3B1 samples using the independent variables sample (S), same plate replication (R), and different plate replication (P).

Source	Sum of squares	df	Mean square	F	SD
Between samples	4.36	9	0.48		0.696
Within samples (S)	0.37	30	0.01	0.012	0.110
Plates (P)	0.00	1	0.00	0.000	
P x S	0.21	9	0.02		
Repetition (R)	0.00	1	0.00	0.000	
R x S	0.03	9	0.003		
P x R	0.03	1	0.03	3.000	
P x R x S	0.10	9	0.01		

df = degrees of freedom
SD = standard deviation

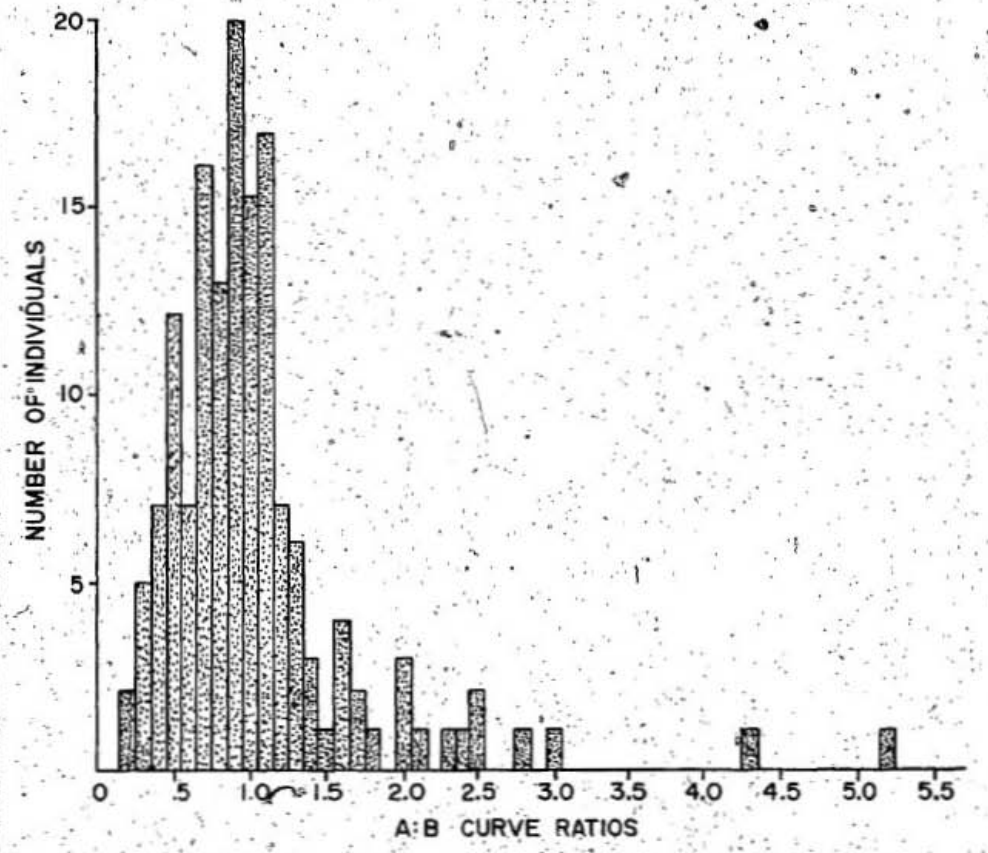


Figure IV-10: Distribution of C4 A:B curve ratios obtained from crossed immunoelectrophoresis of 154 A3B1 plasma samples.

Fifty-nine of the 154 samples tested had A:B ratios of 1.0 ± 20% (+ 2 SD, taking experimental error only), 17 samples had ratios of 0.5 ± 20%, and 11 samples had ratios of 2.0 ± 20%. In other words, there were 67 of 154 samples with A:B ratios somewhere between 0.5 and 1.0, 1.0 and 2.0, and above 2.0. In addition, 59 of 154 samples had A:B ratios of approximately 1.0 and could therefore possess either two or four C4 genes. Since such a large proportion (82%) could not be categorized with certainty by this technique, "conservative" genotypes (i.e. * = "or null") were retained for all non-informative samples.

ii. Families with the rare variants A1 or B3 and A7 or B4

Both A1 or B3 and A7 or B4 have electrophoretic positions intermediate to A3 and B1, with A1 or B3 lying just cathodal to A7 or B4. Since position cannot be used in assigning these to A or B categories, three other criteria were considered, namely, other allelic variants in the family, results of Chido and Rodgers typing, and results of functional haemolytic overlay.

Other allelic variants in the family:

In phenotypes where there are already two A or two B gene products, these variants have been typed A or B based on the assumption of one A and one B gene per chromosome. In practice, these variants have been seen either in phenotypes where there are already two identifiable A genes, or in phenotypes which are non-informative (one A

and one B gene). No family has been seen in which assignment of A (A1, A7) is the only possible interpretation, but there are two families where A7 or B4 must be B4 and three where A1 or B3 must be B3. In all other instances, these variants could be interpreted either way. The variants were assigned the letter B (B3, B4) in all haplotypes, although assignment to the B series for a number of these must be considered tentative.

Results of Chido and Rodgers typing:

Table IV-5 shows the Rodgers typing results of 144 C4-genotyped individuals informative for Rg antigen. A6, A4, A3, and A2 carry Rg antigenic determinants, whereas samples typed AQO/AQO are Rg-. Thirteen individuals, typed Rg (partial inhibitor), are A3, A2, or both (see iii below, p. 112). No individuals were observed with A7 or B4 who were informative for Rg. One individual has A1 or B3 in combination with AQO and B1. This individual is Rg- suggesting that the pattern "A1 or B3" in this individual is in fact B3.

Table IV-6 shows the results of Chido typing 139 C4-genotyped individuals who are informative for this antigen. B variants (B2, B1) carry the Ch antigenic determinants and individuals typed BQO/BQO are Ch-. Ten individuals carry the A7 or B4 allelic product in combination with BQO. Of these, four are Ch+, five are Ch- and one has been tested three times with varying results, either weakly or moderately positive. If A locus products

Table IV-5: Rodgers typing results for C4 genotypes informative for Rodgers antigen.

Rodgers type	A-Locus alleles	C4 genotype	Number observed	Total
Rg-	AQO/AQO	AQOB1/AQOB1	36	
		AQOB1/AQOB2	15	
		AQOB1/AQOB3	1	
				52
Rg+	AQO/A3	AQOB1/A3B1	38	
		AQOB1/A3B1*	12	
		AQOB1/A3BQ0	5	
		AQOB1/A3B3	3	
		AQOB2/A3B1	1	
	AQO/A2	AQOB1/A2B2	4	
		AQOB1/A2B1	2	
		AQOB1/A2B2B1*	1	
	AQO/A4	AQOB1/A4B2	10	
	AQO/A6	AQOB1/A6B1	3	
				79
Rg(part)	AQO/A3	AQOB1/A3BQ0	9	
	AQO/A3A2	AQOB1/A3A2BQ0	3	
		AQOB2/A3A2B2*	1	
				13
Total				144

Table IV-6: Chido typing results for C4 genotypes informative for Chido antigen.

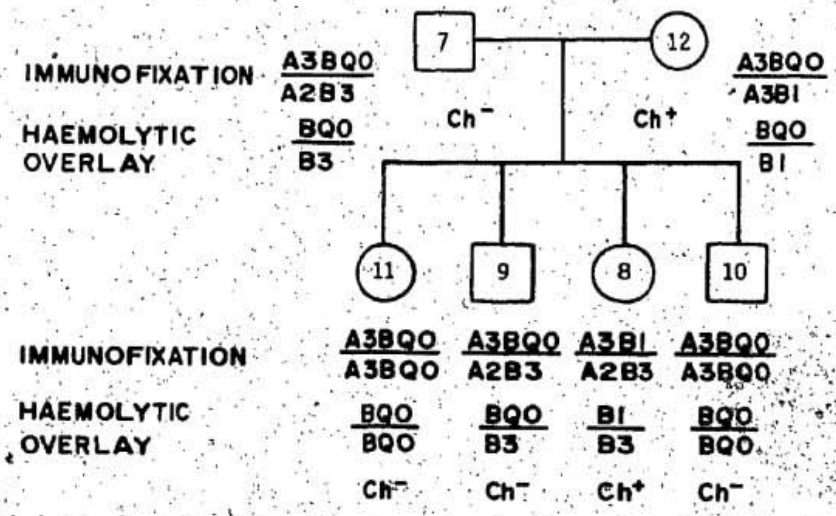
Chido type	B-locus alleles	C4 genotype	Number observed	Total
Ch-	BQ0/BQ0	A3BQ0/A3BQ0	39	
		A3BQ0/A2BQ0	3	
		A3BQ0/A3A2BQ0	1	
	BQ0/B4	A3BQ0/A4B4	5	
	BQ0/B3	A3BQ0/A2B3	2	
		A3BQ0/A3B3	1	
				51
Ch+	BQ0/B1	A3BQ0/A3*B1	16	
		A3BQ0/A3B1	24	
		A3BQ0/AQ0B1	10	
		A3BQ0/A2B1	3	
		A2BQ0/A2B1	1	
		A2BQ0/A3B1	1	
		A3A2BQ0/A3B1	2	
		A3*A2BQ0/A3B1	2	
	BQ0/B2	A3BQ0/A4B2	4	
		A2BQ0/A3B2	2	
		A2BQ0/A3*B2	1	
		A2BQ0/A4B2	3	
		A3BQ0/A3*B2	1	
		A3BQ0/AQ0B2	1	
		A2BQ0/AQ0B2	1	
	BQ0/B3	A3BQ0/A3B3	6	
		A3BQ0/A3*B3	1	
		A3BQ0/A2B3	1	
	BQ0/B4	A3*A2BQ0/A4B4	4	
	BQ0/B2B1	A2BQ0/A2*B2B1	1	
				85
Ch(part)?	BQ0/B3	A3BQ0/A3B3	2	
	BQ0/B4	A3*A2BQ0/A4B4	1	
				3
Total				139

are the ones which carry the Rodgers antigen, and B the Chido, then about half of these A7 or B4 products would be classified as A7, the rest as B4. Thirteen individuals have A1 or B3 combined with BQO. Three are Ch-, eight Ch+, and two gave weakly positive results on repeated typings. Although Ch partial inhibitors are not routinely identified in this laboratory, it seems probable that some or all of these B4 and B3 products are Ch partial inhibitors.

Results of functional haemolytic overlay:

Approximately 100 samples were retested by this method. Although the banding patterns obtained are not as sharply defined as with immunofixation, definite regions of haemolysis are seen (Figure IV-11). Using desialated samples, B bands (B2, B1) are strongly functional whereas A bands (A6, A4, A3, A2) are generally nonfunctional or very weakly so. This distinction is not totally reliable however, since A regions which are non-functional or weakly functional after 20 minutes incubation sometimes show blurred areas of haemolysis after incubation for one hour. B bands always develop first and always within 20 minutes.

All samples containing A1 or B3 and A7 or B4 which were tested showed patterns of strong haemolysis like known B variants. One family with B3 is illustrated in Figure IV-11. Immunoprecipitation and haemolytic patterns are included for comparison. Sample #7 and sample #9 have B3 together with BQO. Both are Ch-. The B3 is functional in



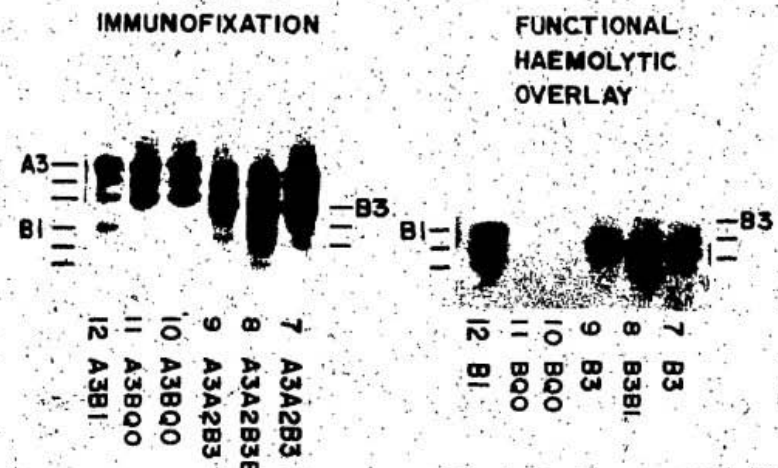


Figure IV-11: A family which illustrates segregation of the C4 haplotypes A3BQO, A2B3, and A3B1. C4A gene products are non-functional in haemolytic overlay. The B3 variant is Ch-. Phenotypes are given below the photographs.

haemolytic overlay and is most likely a product of the B locus since, if it is an A allelic product, individuals 7, 8, and 9 must have three A genes.

On the basis of functional overlay where tested, and family segregation where informative, A1 or B3 and A7 or B4 behave as if they were B-allelic products. It must be emphasized, however, that all individuals with these products do not come from families informative for Ch and/or C4.

iii. Families with A3 and A2 together and B1 and B2 together

Individuals with phenotypes containing A3 and A2 are difficult to interpret. In some cases individuals can be genotyped A3/A2 since the two products were clearly seen to segregate to different haplotypes in the family. One such family is illustrated in Figure IV-9. In other families, A3 and A2 appear not to segregate, that is, there are individuals who appear to have two A genes on the same haplotype. Two pedigrees with A3A2 haplotypes are illustrated in Figures IV-12 and IV-13. In the family shown in Figure IV-12, individual #328 with phenotype A3A2B2 has inherited haplotype b, AQOB2, from his mother. In the family shown in Figure IV-13, individual #245 has given haplotype e, A3A2BQO, to two daughters and a son. The spouse (#217) of one daughter also has A3A2BQO and their child, #249, is homozygous A3A2BQO/A3A2BQO.

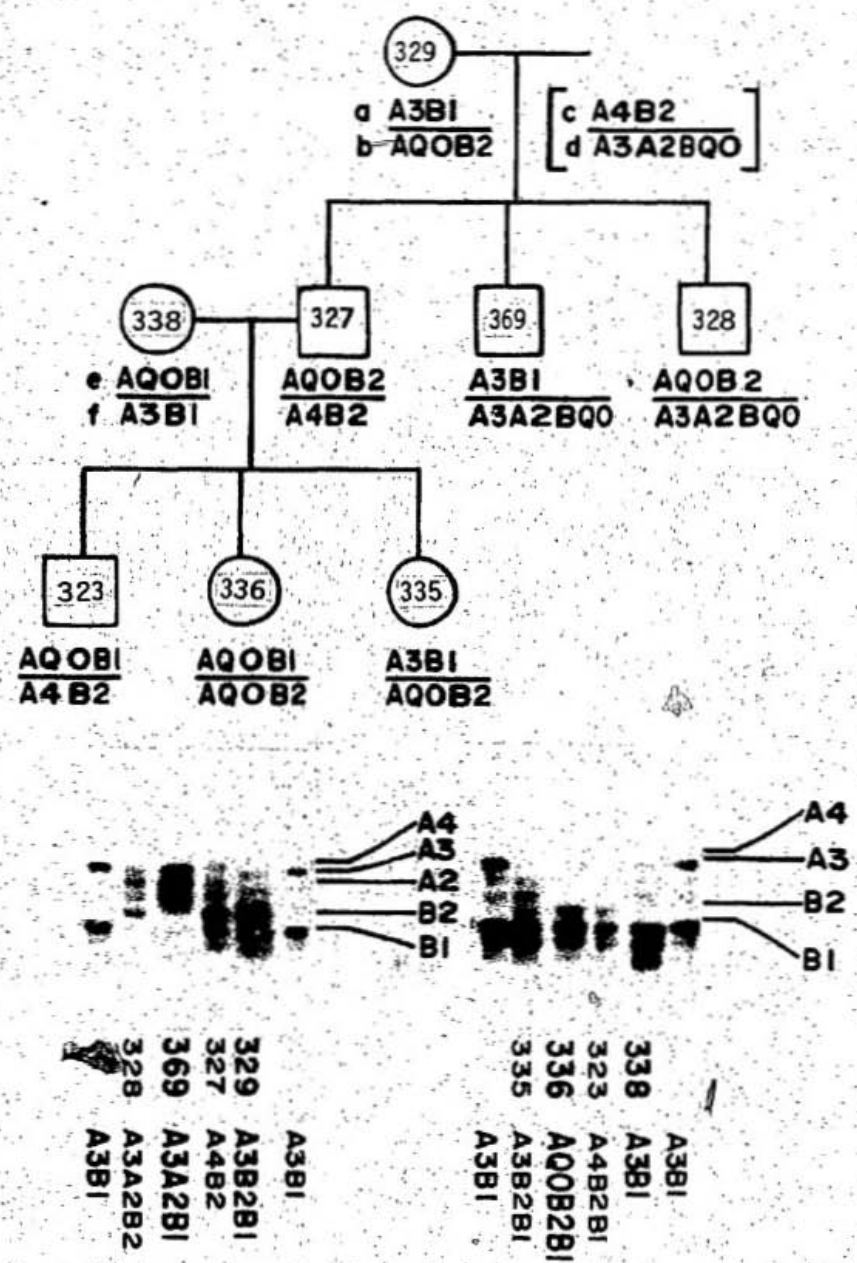


Figure IV-12: A family which illustrates segregation of the C4 haplotypes A3B1, AQOB2, A4B2, and A3A2BQO. Entry haplotypes are designated a, b, c, and d. Phenotypes are given below the photograph.

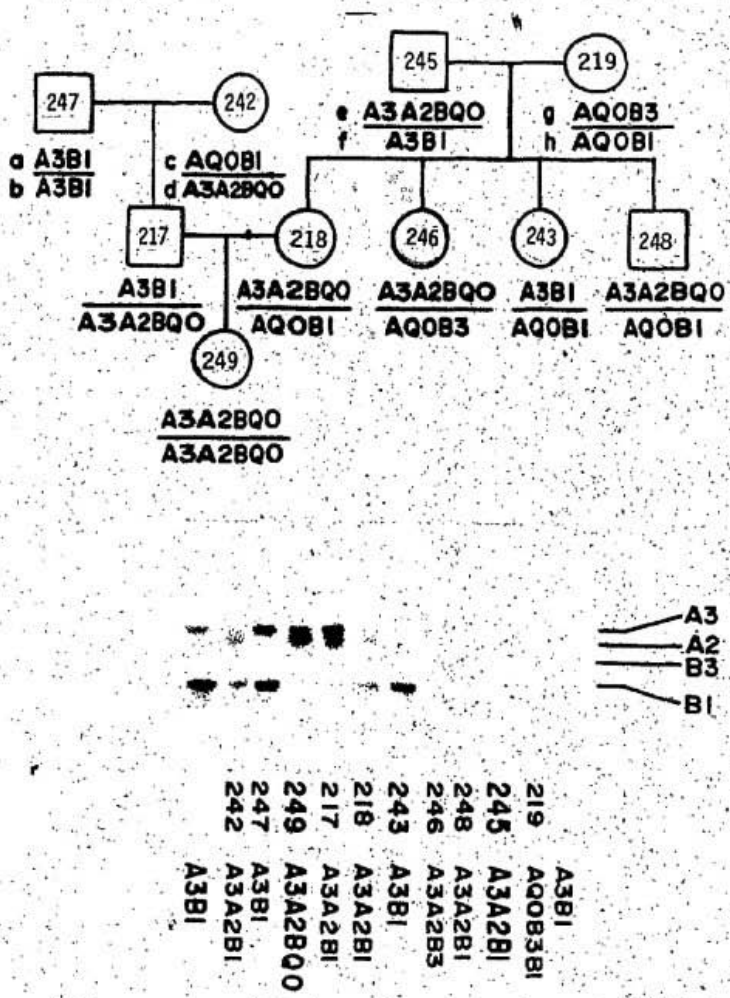


Figure IV-13: A family which illustrates segregation of the C4 haplotypes A3B1, AQOB1, A3A2BQO, and AQOB3. Entry haplotypes are designated a, b, c, d, etc. Phenotypes are given below the photograph.

There are a few partially informative families in which a A3A2 haplotype, though plausible, cannot be demonstrated with certainty. In these cases an individual was assigned the haplotype A3*A2 if his other haplotype was known to contain A3, and A3A2* if the other haplotype was known to contain A2. No A3A2 haplotype has been seen with a demonstrable gene product at the B locus. In other words, all are A3A2BQO, A3A2B1*, or A3A2B2*.

The evidence for two B genes on a single haplotype is less secure. In three families with B2B1 patterns, the families cannot be interpreted on a single B gene per chromosome assumption unless one hypothesizes, in each family, a recombinant in the MHC region. Only C4 is at all informative. The C4 patterns of each family can be readily interpreted if one assumes that some individuals have a haplotype containing both B2 and B1 alleles. There are four of these haplotypes in three families. All four have the same HLA-C and -B alleles. At least two have the same DR. (The other two were not tested). The pedigree of one of these families is illustrated in Figure IV-14. All four putative "double B" haplotypes have A2 at the A locus. A haplotype was called A2B2B1* if it was found only in genotypes where the other haplotype was known to contain B1 and A2B1B2* if it was found only in genotypes where the other haplotype was known to contain B2. The family shown in Figure IV-14 contains two individuals who, if the A2B2B1-haplotype interpretation is correct, must be

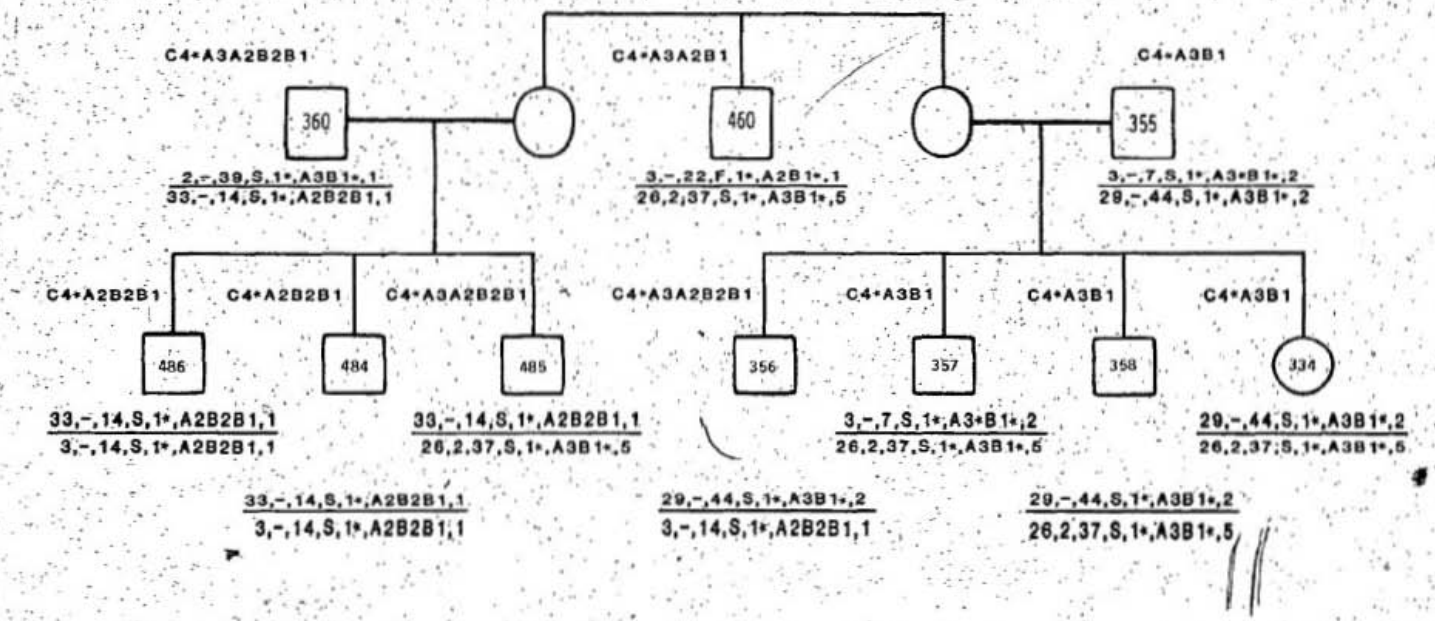


Figure IV-14: A family with two putative "double B" or A2B2B1 haplotypes. C4 phenotypes are given above and suggested haplotypes below the pedigree symbols. The order of alleles in each haplotype is HLA-A, HLA-C, HLA-B, BF, C2, C4, and HLA-DR.

homozygous. A2B2B1/A2B2B1.

d. Haplotypes

From the six sources of family material, 1048 founder or entry haplotypes were extracted. Of these, 603 are "complete" haplotypes which can be definitely assigned a C4 allele at each of the two loci. Fifteen different complete C4 haplotypes have been observed. The numbers observed and their relative frequencies are given in Table IV-7. Six haplotypes, A3B1, AQOB1, A3BQO, A4B2, A6B1, A4B4, appeared with a frequency greater than 2% and account for 92% of the total.

The middle six columns of Table IV-7 contain the numbers and relative frequencies of haplotypes observed in each of the sources of family material. Comparing haplotype frequencies from the four larger sources, FS, MS, WC, WP, it can be seen that five of the six common haplotypes vary in frequency from one source to another. 45% of the MS haplotypes are A3B1 as compared to 32% of WC. 13% of the WC haplotypes are AQOB1 compared with 20% of the total. A3BQO haplotypes constitute 27% of WC but 10% of MS. A6B1 is increased in the WC sample with 10% as compared to 4% of the total.

These frequencies are distorted however, as can be seen from Table IV-8 and Figure IV-15. Table IV-8 shows the remaining 445 haplotypes observed. These are indefinite or

Table IV-7: Numbers and frequencies of completely-typed C4 entry haplotypes observed in six sources of family material.

C4 haplotype	FS	LA	MS	MV	WC	WP	Total	Frequency
A3B1	49	11	112	4	32	28	236	.3914
AQOB1	28	6	47	5	22	10	118	.1957
A3BQO	22	2	24	2	27	12	89	.1476
A4B2	15	1	32	3	12	10	73	.1211
A6B1	14	0	8	1	0	2	25	.0415
A4B4	3	0	5	2	1	4	15	.0249
AQOB2	1	1	3	0	1	3	9	.0149
A2B2	1	0	5	2	1	0	9	.0149
A2B1	0	0	4	0	2	2	8	.0133
A3B3	2	0	5	0	0	1	8	.0133
A2BQO	1	0	3	0	0	2	6	.0100
A3A2BQO	0	0	0	0	1	2	3	.0050
A3B2	0	0	2	0	0	0	2	.0033
AQOB3	0	0	0	0	0	1	1	.0017
A2B3	0	0	1	0	0	0	1	.0017
Total	136	21	251	19	99	77	603	1.0000

Table IV-8: Numbers and frequencies of partially-typed or "incomplete" entry haplotypes from six sources of family material.

C4 haplotype	FS	LA	MS	MV	WC	WP	Total	Frequency
A3*B1*	59	0	34	10	31	38	172	.3865
A3B1*	34	3	30	2	18	16	103	.2315
A3*B1	29	0	23	5	32	9	98	.2202
A2B1*	4	0	4	0	1	3	12	.0270
A3*B3	6	0	0	0	4	1	11	.0247
A3*B2	4	0	6	0	0	0	10	.0225
A3*A2BQO	2	0	7	0	1	0	10	.0225
A2B2B1*	1	0	4	0	1	1	7	.0157
A3*A2B1*	1	0	2	0	0	4	7	.0157
A2*A3BQO	0	0	0	0	4	1	5	.0112
A2B1B2*	0	0	2	0	0	1	3	.0067
A2*B1	0	0	0	0	2	0	2	.0045
A2B2*	0	0	1	0	0	0	1	.0022
A3B3*	0	0	0	0	1	0	1	.0022
A4*B2*	0	0	1	0	0	0	1	.0022
A2*B2B1*	0	0	0	0	1	0	1	.0022
A3*A2B2*	0	0	1	0	0	0	1	.0022
Total	140	3	115	17	96	74	445	1.0000

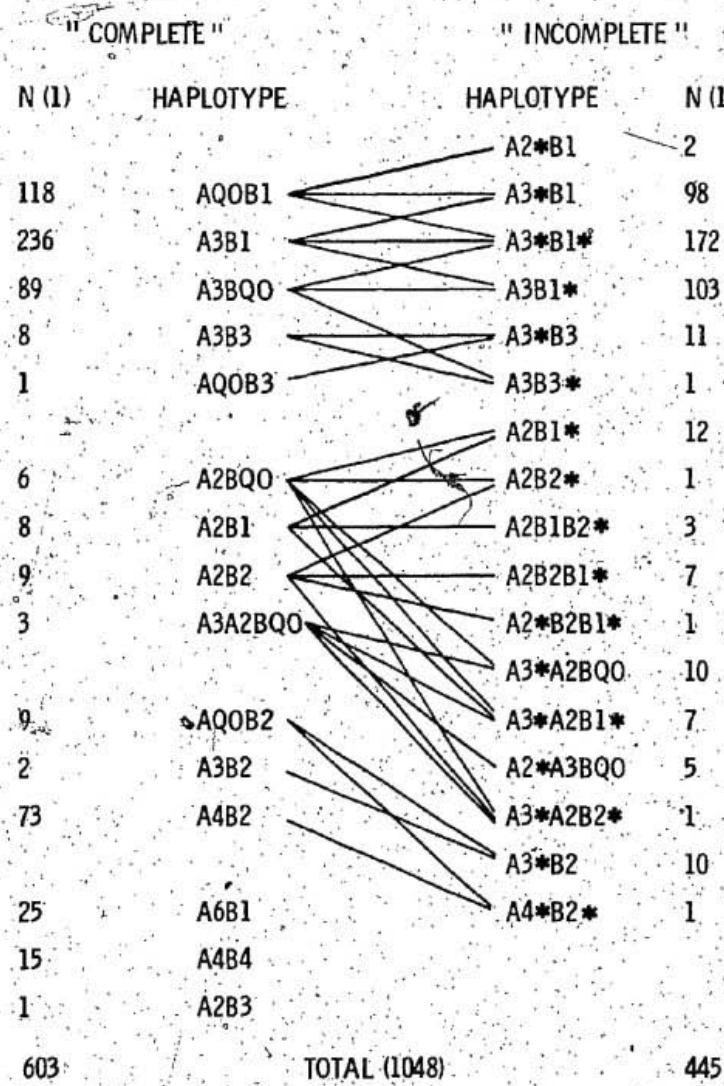


Figure IV-15: The relationship between "complete" and "incomplete" haplotypes. Lines connect incomplete haplotypes on the right with alternate interpretations on the left.

"incomplete" since at least one allele in each (*) can be alternatively interpreted as null. Of the 445 incomplete haplotypes, 39%, 23%, and 22% are A3*B1*, A3B1*, and A3*B1 respectively. Seven haplotypes, A3*A2BQO, A3*A2B1*, A2*A3BQO, A3*A2B2*, A2B2B1*, A2*B2B1*, and A2B1B2*, accounting for 8% of the total, are likely to have two A or two B genes per chromosome.

Figure IV-15 shows the relationship between complete and incomplete haplotypes. There are, for example, 118 complete AQOB1 haplotypes and 98 incomplete A3*B1, 172 A3*B1*, and two A2*B1. Any or all of the latter three may be interpreted as AQOB1, i.e. the true frequency of AQOB1 falls between a minimum of 0.113 and a maximum of 0.372. Another example is A2BQO. There are only six definite A2BQO haplotypes, but 31 incomplete, including A2B1*, A2B2*, A3*A2BQO, A3*A2B1*, and A3*A2B2*. Any of these may be A2BQO.

Table IV-9 combines the data from Tables IV-7 and IV-8. All haplotypes observed and their relative frequencies in each population sample (FS, LA, etc.) and in the total (1048 haplotypes) are listed. Of the figures given for the fifteen complete haplotypes, only four accurately reflect their true proportions. As can be seen from Figure IV-15, 25 A6B1, 73 A4B2, 15 A4B4, and one A2B3 have been definitely characterized since none of the incomplete haplotypes can be A6B1, A4B4, or A2B3. Only one,

Table IV-9: Total 04 entry haplotypes identified in 1857 individuals from six sources of family material. Frequencies for each source are also shown.

C4 haplotype	FS	LA	MS	MV	WC	WP	Total
A3B1	49	11	112	4	32	28	236
	.1775	.4583	.3060	.1111	.1641	.1854	.2252
AQOB1	28	6	47	5	22	10	118
	.1014	.2500	.1284	.1389	.1128	.0662	.1126
A3BQO	22	2	24	2	27	12	89
	.0797	.0833	.0656	.0556	.1385	.0795	.0849
A4B2	15	1	32	3	12	10	73
	.0543	.0417	.0874	.0833	.0615	.0662	.0697
A6B1	14	0	8	1	0	2	25
	.0507		.0219	.0278		.0132	.0239
A4B4	3	0	5	2	1	4	15
	.0109		.0137	.0556	.0051	.0265	.0143
AQOB2	1	1	3	0	1	3	9
	.0036	.0417	.0082		.0051	.0199	.0086
A2B2	1	0	5	2	1	0	9
	.0036		.0137	.0556	.0051		.0086
A2B1	0	0	4	0	2	2	8
			.0109		.0103	.0132	.0076
A3B3	2	0	5	0	0	1	8
	.0072		.0137			.0066	.0076
A2BQO	1	0	3	0	0	2	6
	.0036		.0082			.0132	.0057
A3A2BQO	0	0	0	0	1	2	3
					.0051	.0132	.0029
A3B2	0	0	2	0	0	0	2
			.0055				.0019
AQOB3	0	0	0	0	0	1	1
						.0066	.0010

Table IV-9: continued.

C4 haplotype	FS	LA	MS	MV	WC	WP	Total
A2B3	0	0	1 .0027	0	0	0	1 .0010
A3*B1*	59 .2138	0	34 .0929	10 .2778	31 .1590	38 .2517	172 .1641
A3B1*	34 .1232	3 .1250	30 .0820	2 .0556	18 .0923	16 .1060	103 .0983
A3*B1	29 .1051	0	23 .0628	5 .1389	32 .1641	9 .0596	98 .0935
A2B1*	4 .0145	0	4 .0109	0	1 .0051	3 .0199	12 .0115
A3*B3	6 .0217	0	0	0	4 .0205	1 .0066	11 .0105
A3*B2	4 .0145	0	6 .0164	0	0	0	10 .0095
A3*A2BQO	2 .0072	0	7 .0191	0	1 .0051	0	10 .0095
A2B2B1*	1 .0036	0	4 .0109	0	1 .0051	1 .0066	7 .0067
A3*A2B1*	1 .0036	0	2 .0055	0	0	4 .0265	7 .0067
A2*A3BQO	0	0	0	0	4 .0205	1 .0066	5 .0048
A2B1B2*	0	0	2 .0055	0	0	1 .0066	3 .0029
A2*B1	0	0	0	0	2 .0103	0	2 .0019
A2B2*	0	0	1 .0027	0	0	0	1 .0010

Table IV-9: continued.

C4 haplotype	FS	LA	MS	MV	WC	WC	Total
A4*B2*	0	0	1 .0027	0	0	0	1 .0010
A3*B2B1*	0	0	0	0	1 .0051	0	1 .0010
A3*A2B2*	0	0	1 .0027	0	0	0	1 .0010
A3B3*	0	0	0	0	1 .0051	0	1 .0010
Total	276 1.000	24 1.000	366 1.000	36 1.000	195 1.000	151 1.000	1048 1.000

A4*B2*, can be A4B2.

e. Gene frequencies

The frequencies of C4*A and C4*B alleles were obtained by direct count from the haplotype data. Table IV-10 shows a total A-gene count of 1074, which includes 26 extra genes from haplotypes postulated to have two A genes per haplotype. Of the total, 42% is A3, 12% is AQO, and 29% is A3*, that is, AQO or A3. No A6* and only seven A2* and one A4* were observed. The figures given for A4, A6, and A2 can therefore be taken as fairly accurately representing the true frequencies of these alleles in the sample.

Eleven of the total C4*B alleles given in Table IV-11 come from haplotypes postulated to have two B genes per haplotype. The most frequently occurring B alleles are B1 and BQO with 46% B1, 11% BQO, and 29% B1*, that is, B1 or BQO. There are no B4* and only six B2* and one B3*, so the frequencies given for these alleles (10% B2, 2% B3, and 1.4% B4) can be taken as accurately reflecting their true frequencies in the total sample.

The relationships between A3, AQO, and A3* alleles, and between B1, BQO, and B1* alleles, were examined to determine if the relative frequencies of A3 and AQO, and of B1 and BQO, could be used to estimate the proportions of these alleles represented by A3* and B1*. From Table IV-10 it can be seen that 447 or 78% of the 575 known A3 + AQO

Table IV-10: Frequencies of C4A alleles obtained by direct count from entry haplotypes.(1)

Allele	FS	LA	MS	MV	WC	WP	TOTAL
AQO	29	7	50	5	23	14	128
	.1039	.2917	.1330	.1389	.1144	.0886	.1192
A2	10	0	34	2	7	15	68
	.0358		.0904	.0556	.0348	.0949	.0633
A3	107	16	173	8	83	60	447
	.3835	.6667	.4601	.2222	.4129	.3797	.4162
A4	18	1	37	5	13	14	88
	.0645	.0417	.0984	.1389	.0647	.0886	.0819
A6	14	0	8	1	0	2	25
	.0502		.0213	.0278		.0127	.0233
A2*	0	0	0	0	6	1	7
					.0299	.0063	.0065
A3*	101	0	73	15	69	52	310
	.3620		.1941	.4167	.3433	.3291	.2886
A4*	0	0	1	0	0	0	1
			.0027				.0009
Total	279	24	376	36	201	158	1074

(1) Includes alleles from 26 entry haplotypes which may have two A alleles per haplotype.

Table IV-11: Frequencies of C4B alleles obtained by direct count from entry haplotypes. (1)

Allele	FS	LA	MS	MV	WC	WP	TOTAL
BQ0	25	2	34	2	33	17	113
	.0903	.0833	.0914	.0556	.1675	.1111	.1067
B1	120	17	196	15	90	52	490
	.4332	.7083	.5269	.4167	.4569	.3399	.4627
B2	22	2	52	5	16	14	111
	.0794	.0833	.1398	.1389	.0812	.0915	.1048
B3	8	0	6	0	4	3	21
	.0289		.0161		.0203	.0196	.0198
B4	3	0	5	2	1	4	15
	.0108		.0134	.0556	.0051	.0261	.0142
B1*	99	3	74	12	52	62	302
	.3574	.1250	.1989	.3333	.2640	.4052	.2852
B2*	0	0	5	0	0	1	6
			.0134			.0065	.0057
B3*	0	0	0	0	1	0	1
					.0051		.0009
Total	277	24	372	36	197	153	1059

(1) Includes alleles from 11 entry haplotypes assumed to have 2 B alleles per haplotype.

alleles are A3 and 128 or 22% are AQO. Applying these proportions to A3* alleles gives 242 A3 and 68 AQO. The estimated frequencies of these genes become, for A3, 447 + 242 = 689 or 64%, and for AQO, 128 + 68 = 378 or 18%. Similarly, one can estimate the frequency of B1 (from Table IV-11) to be 490 + (490/603 X 302) = 735 or 69% and of BQO to be 113 + (113/603 x 302) = 170 or 16%.

These estimates must be treated with caution however. Table IV-12 shows the results of chi-square analysis of the relative frequencies of (a) A3, AQO, and A3* and (b) B1, BQO, and B1* in each of the sources sampled. For both A and B genes, the proportions of the three alleles vary significantly in the six series examined.

f. Associations between A and B gene products

Of the 25 two-gene haplotypic combinations possible among five A and five B allelic variants, only 14 were observed. Associations between C4*A and C4*B alleles are given in Table IV-13. Putative "double" haplotypes were treated as separate categories in this analysis.

Nine haplotypes have high positive delta values which are significant at the 0.05% level. Thirteen combinations show strong negative associations and of these, 10 are highly significant. Significantly associated pairs are listed in Table IV-14.

No family was observed in which the existence of an

Table IV-12: Comparison of observed and expected C4*A and C4*B allele frequencies in the five sources sampled.

ALLELE		FS	LA	MS	MV	WC	WP	TOTAL
B1	o	120	17	196	15	90	52	490
	e	132.11	11.91	164.60	15.70	94.75	70.93	
BQO	o	25	2	34	2	33	17	113
	e	30.47	2.75	37.96	3.62	21.85	16.36	
B1*	o	99	3	74	12	52	62	302
	e	81.42	7.34	101.45	9.68	58.40	43.71	
TOTAL								
	o	244	22	304	29	175	131	905

$\chi^2 = 45.34$
10 df
$p < .05$

A3	o	107	16	173	8	83	60	447
	e	119.71	11.62	149.51	14.14	88.39	63.64	
AQO	o	29	7	50	5	23	14	128
	e	34.28	3.33	42.81	4.05	25.31	18.22	
A3*	o	101	0	73	15	69	52	310
	e	83.02	8.06	103.68	9.81	61.30	44.14	
TOTAL								
	o	237	26	296	28	175	126	

$\chi^2 = 43.52$
10 df
$p < .05$

Table IV-13: Linkage disequilibrium values for C4*A- and C4*B-gene pairs. Number observed (o), delta ($\Delta \times 10^4$) and χ^2 are shown for pairs where number expected $\geq$ 2, except where indicated. All $\chi^2 > 12.12$ are significant at the 0.05% level and all $\chi^2 > 7.9$ are significant at the 5% level.

		BQ0	B1	B2	B3	B4	B1*	B2*	B3*	B2B1 (1)	Total
AQ0	o	0	118	9	1	0	0	0	0	0	128
	Δ	-132	+559	-34	-14		-342				
	χ^2	16.4	120.4	1.0	1.9		55.3				
A2	o	6	8	9	1	0	12	1	0	10(2)	47
	Δ	+9	-132	+42			-11			+90	
	χ^2	0.0	15.9	3.8			0.5			174.0	
A3	o	89	236	2	8	0	103	0	1	0	439
	Δ	+397	+304	-393	-8	-60	-192			-440	
	χ^2	69.1	15.6	73.1	0.0	9.3	7.5			6.4	
A4	o	0	0	73	0	15(2)	0	0	0	0	88
	Δ	-91	-390	+614		+131	-236				
	χ^2	10.2	81.4	570.7		154.2	36.0				
A6	o	0	25	0	0	0	0	0	0	0	25
	Δ	-25	+128	-23			-67				
	χ^2	2.1	27.3	1.8			8.6				
A2*	o	0	2	0	0	0	0	0	0	1	3
A3*	o	0	98	10	11	0	172	0	0	0	291
	Δ	-299	-354	-178	+49	-40	+862				
	χ^2	47.2	25.8	17.6	5.3	4.5	190.4				
A4*	o	0	0	0	0	0	0	1	0	0	1
A3A2 (3)	o	18	0	0	0	0	7	1	0	0	26
	Δ	+145	-115	-24			-3				
	χ^2	42.8	21.3	1.9			0				
Total		113	487	103	21	15	294	3	1	11	1048

(1) Includes B2B1* and B1B2*.
(2) Number expected < 1.
(3) Includes A3A2, A3*A2, and A2*A3.

Table IV-14: Non-randomly associated combinations of C4*A and C4*B alleles ($p < .0005$).

Positively associated	Negatively associated
AQOB1	AQOBQO
A3BQO	AQOB1*
A3B1	A2B1
A4B2	A3B2
A4B4	A4B1
A6B1	A4B1*
A3*B1*	A3*BQO
A3A2BQO	A3*B1
A2B2B1	A3*B2
	A3A2B1

AQ0B0 haplotype was the only possible interpetation, nor has any C4-deficient individual been observed. There are clearly preferred haplotypic combinations. AQ0B1 and A3BQ0 have high positive deltas, as does, to a lesser extent, A3B1. A4 occurs only in A4B4 and A4B2 haplotypes and A6 only in A6B1. Negative associations, between A4 and B1, for example, reflect these preferred combinations. The variant A2 is particularly interesting since it occurs less often than expected with B1 and has been observed in a variety of haplotypes, e.g. A2BQ0, A2B1, A2B2, A3A2BQ0, and A2B2B1.

B. Description and analyses of extended MHC haplotypes

1. Recombinant haplotypes

Only four recombinant families informative for C4 were observed. There were three HLA-A/HLA-B recombinant haplotypes and one HLA-B/HLA-DR. The families with A-B breaks are shown in Figures IV-16 to IV-18. In all three cases C4 segregates with the HLA-B segment of the chromosome, away from HLA-A. Family 61 (Figure IV-16) is also informative for BF, while family 22 (Figure IV-17) is also informative for C2. In both cases the alleles segregate with C4/B/DR. Family 251 (Figure IV-19) has an HlA-B/HLA-DR recombinant and is informative for C2 and C4. Both of these complement alleles segregate with the DR portion of the chromosome.

FAMILY 61

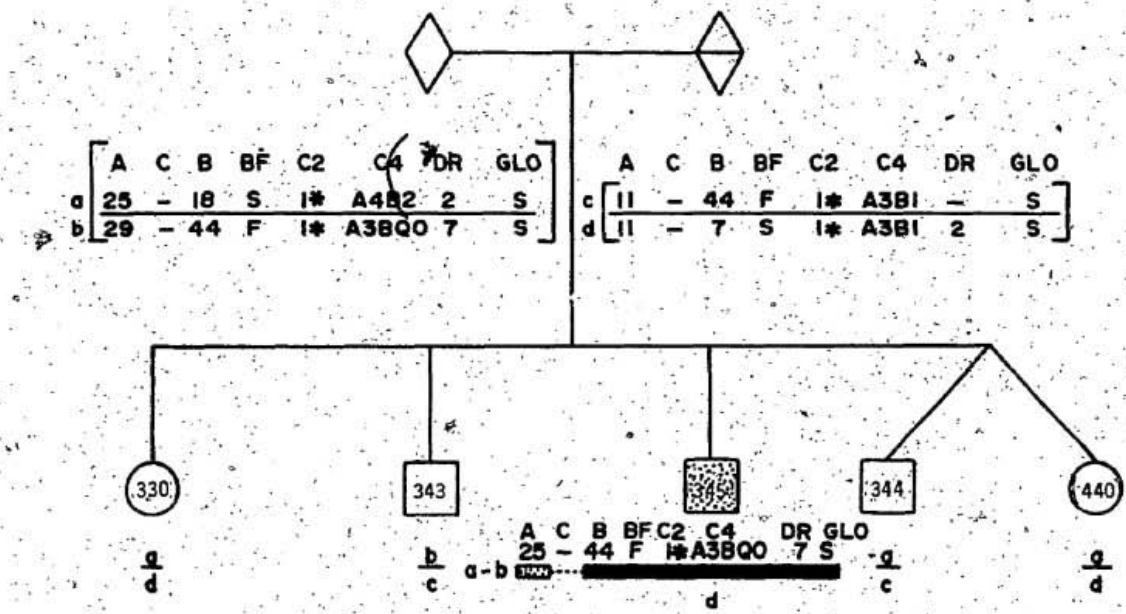


Figure IV-16: A family with recombination between HLA-A and HLA-B, informative for BF and C4. Entry haplotypes are indicated as a,b,c,d.

FAMILY 22

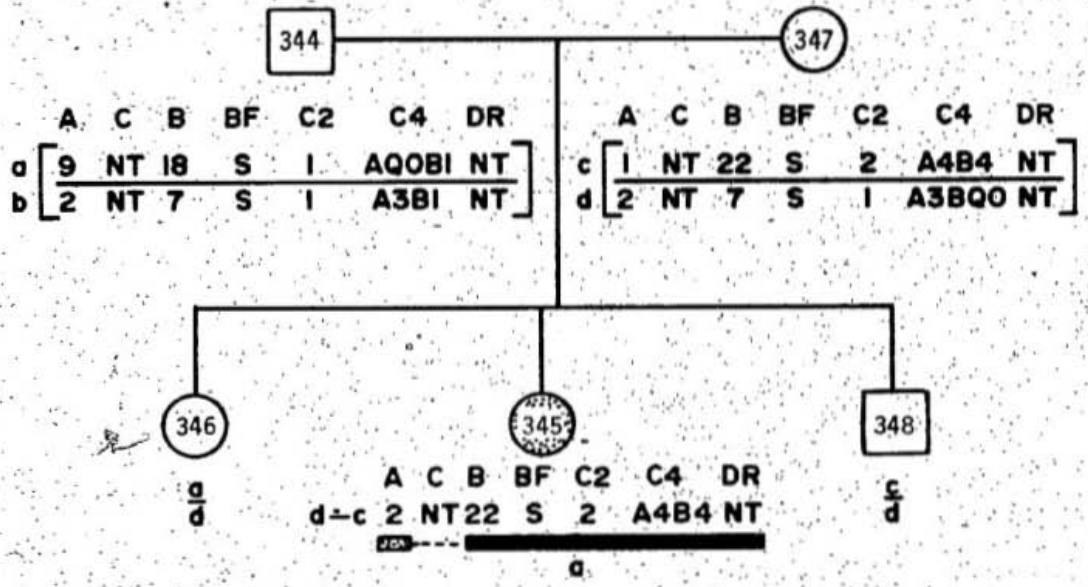


Figure IV-17: A family with a maternal recombination between HLA-A and HLA-B, informative for C2 and C4. Entry haplotypes are designated a, b, c, d.

FAMILY 70

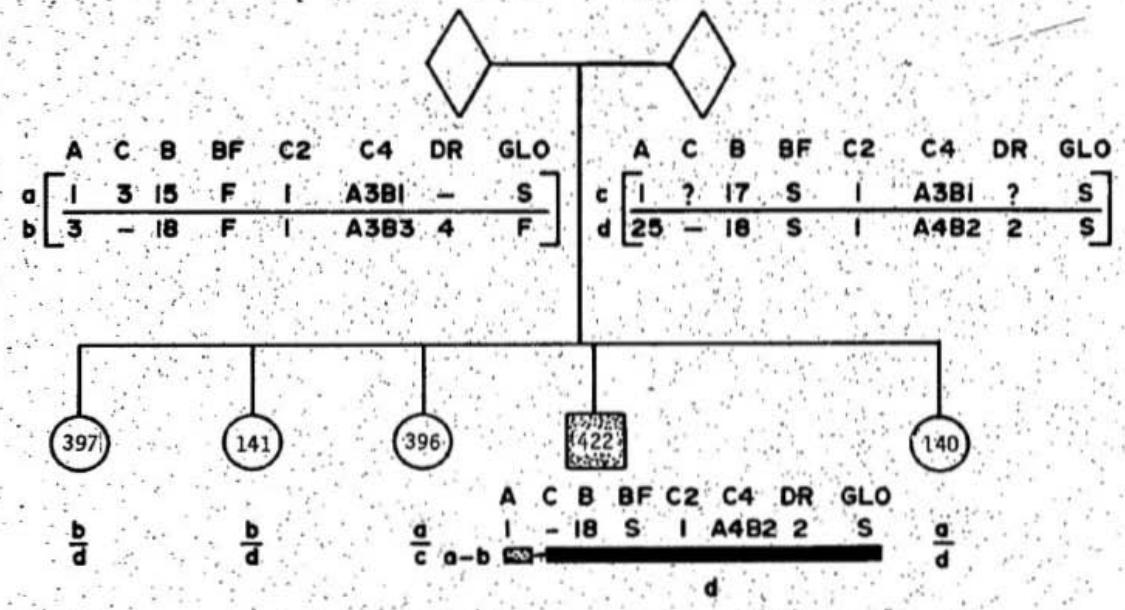


Figure IV-18: A family with a recombination between HLA-A and HLA-B informative for C4. Entry haplotypes are designated a, b, c, d.

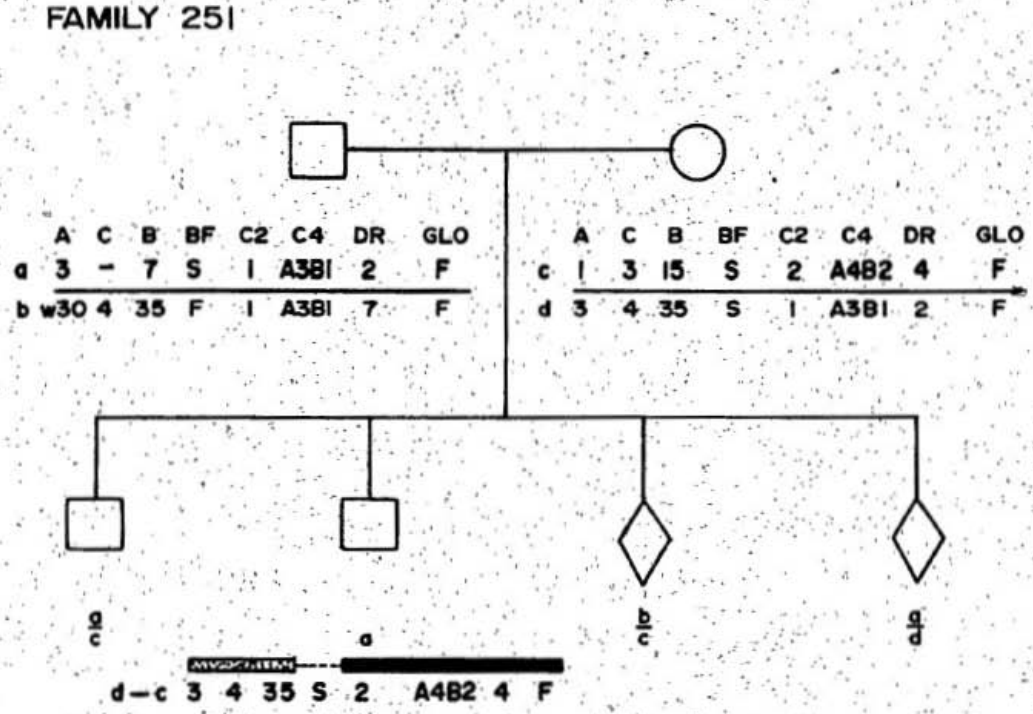


Figure IV-19: A family illustrating a maternal recombination between HLA-B and HLA-DR informative for C4 and C2. Entry haplotypes are designated a, b, c, d.

2. Frequencies of other MHC antigens

Tables IV-15 to IV-18 show the frequencies of the MHC alleles (except C4) in the various study populations. These frequencies were determined by direct count from entry haplotypes. Also shown, for comparison, in Tables IV-17 and IV-18 are the frequencies of HLA-B alleles obtained from the pooled data at the 1980 Histocompatibility Workshop (Bauer and Danilovs, 1980). It can be seen that the frequencies for HLA-B alleles in the study populations differ from the Workshop Caucasoid frequencies primarily for HLA-B7, B8, and B12. B7 is increased in all four of the larger study populations, and most particularly in the MS population. B8 is increased in all four of the larger groups studied, while B12 is more frequent in the WC group. The frequencies of BF and C2 variants do not differ markedly from published figures. Only the MS population was completely typed for HLA-DR. It can be seen from Table IV-18 that DR2, DR3, and DR4 appear to be increased in this population.

3. Associations among MHC alleles

Two-way associations were calculated by both chi square and delta standard methods for (a) all pairs of HLA-B, -DR, and BF and C2 alleles, and (b) all pair-wise combinations of C4 haplotypes and HLA-B, -DR, and BF and C2

Table IV-15: Numbers and frequencies of BF alleles in six study populations.

Allele		FS	LA	MS	MV	WC	WP	Total
BF	S	199	19	287	28	138	99	770
		.85	.83	.82	.78	.77	.74	.80
	S1	2	0	3	0	3	3	11
		.01	.00	.01	.00	.03	.02	.01
	F	33	3	59	8	37	32	172
		.14	.13	.17	.22	.21	.24	.18
	F1	1	1	2	0	2	0	6
		.00	.04	.01	.00	.01	.00	.01
Total		235	23	351	36	180	134	959
		1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table IV-16: Numbers and frequencies of C2 alleles.

Allele	FS	LA	MS	MV	WC	WP	Total
C2*1* (1)	174	17	334	34	177	117	853
	.95	1.00	.97	.97	.95	.95	.96
C2*2	10	0	12	1	9	6	38
	.05	.00	.03	.03	.05	.05	.04
Total	184	17	346	35	186	123	891
	1.00	1.00	1.00	1.00	1.00	1.00	1.00

(1) Includes C2*1 and 1*.

Table IV-17: Numbers and frequencies of HLA-B alleles in the six study populations with North American and European Caucasoid frequencies given for comparison.

HLA	FS	LA	MS	MV	WC	WP	Total	NAC*	EUC*
-	3	0	4	0	0	0	7		
	.01	.00	.01	.00	.00	.00	.01	.02	.06
5	20	4	13	2	7	13	59		
	.08	.17	.04	.06	.04	.08	.06	.06	.09
7	37	0	78	5	35	19	174		
	.14	.00	.22	.14	.18	.12	.17	.10	.09
8	39	5	48	6	24	14	136		
	.15	.21	.14	.17	.12	.09	.13	.09	.08
12	42	2	40	2	44	30	160		
	.16	.08	.11	.06	.23	.19	.16	.15	.12
13	3	0	2	1	1	1	8		
	.01	.00	.01	03	.01	.01	.01	.03	.03
14	11	1	19	2	4	3	40		
	.04	.04	.05	.06	.02	.02	.04	.05	.03
15	17	3	21	3	12	12	68		
	.07	.13	.06	.09	.06	.08	.07	.06	.06
16	6	0	10	2	8	3	29		
	.02	.00	.03	.06	.04	.02	.03	.05	.05
17	15	0	11	1	0	1	28		
	.06	.00	.03	.03	.00	.01	.03	.09	.04
18	9	2	17	0	13	9	50		
	.03	.08	.05	.00	.07	.06	.05	.05	.06
21	1	0	8	0	0	4	13		
	.00	.00	.02	.00	.00	.03	.01	.04	.04
22	6	0	10	1	2	6	25		
	.02	.00	.03	.03	.01	.0.04	.02	.03	.03
27	5	1	16	2	5	8	37		
	.02	.04	.05	.06	.03	.05	.04	.04	.04

continued

Table IV-17: continued.

HLA	FS	LA	MS	MV	WC	WP	TOTAL	NAC*	EUC*
35	20	2	28	4	20	14	88		
	.08	.08	.08	.11	.10	.09	.08	.09	.10
37	3	0	4	1	3	1	12		
	.01	.00	.01	.03	.02	.01	.01	.02	.02
40	18	1	20	1	14	18	72		
	.07	.04	.06	.03	.07	.11	.07	.07	.05
41	3	3	0	2	0	1	9		
	.01	.13	.00	.06	.00	.01	.01	.02	.01
42	0	0	4	0	1	0	5		
	.00	.00	.01	.00	.01	.00	.001	.03	.03
Total	258	24	353	35	193	157	1020		
	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

* From Bauer and Danilovs (1980).

Table IV-18: Numbers and frequencies of HLA-DR alleles observed in MS family and other haplotypes with North American and European Caucasoid frequencies included for comparison.

HLA-DR	MS	Other	Total	NAC*	EUC*
-	30	2	32		
	.09		.09	.30	.18
1	38	0	38		
	.11		.10	.07	.11
2	75	5	80		
	.22		.22	.13	.14
3	57	7	64		
	.16		.17	.11	.12
4	62	5	67		
	.18		.18	.10	.15
5	33	0	33		
	.09		.09	.10	.10
6	5	1	6		
	.01		.02	.02	.04
7	46	0	46		
	.13		.13	.13	.13
8	1	0	1		
	.00		.00	.03	.03
9	1	0	1		
	.00		.00	.01	.02
10	0	0	0		
	.00		.00	.01	.02
Total	348	20	368		

* From Bauer and Danilovs (1980)

alleles. In addition, the calculations elaborated by Dausset (1978) and Grange et al. (1981) were applied to the complete haplotype data to determine whether (c) three-way combinations of C4 haplotypes and the alleles of the complement components BF and C2, and (d) three-, four-, and five-way combinations of C4 haplotypes and HLA-B, -DR, BF and C2 alleles were occurring more frequently than might be expected from their individual antigen frequencies.

Because complete haplotype data on MHC antigens were not available for the total 1048 haplotypes considered in this study, the N value, or the number of haplotypes considered, is different for each calculation. For example, 897 haplotypes were typed for HLA-B and C2 antigens and N is therefore equal to 897 in calculating allele and haplotype frequencies for B-C2 associations. All frequency tables used for these calculations are given in Appendix A, Tables A-1 to A-17. Table IV-19, this chapter, is a guide to those frequency tables. In addition, a complete list of haplotypes is shown in Appendix B. Positive and negative non-random associations are given in Tables IV-20 to IV-28 in which N represents the total haplotypes considered for each calculation and p is the probability obtained by Fisher's exact test. Positive associations are shown for only those haplotypes with observed frequencies ≥ 3 whereas negative associations are given for haplotypes with expected frequencies ≥ 3.

Table IV-19: Guide to the frequency tables in Appendix A.

MHC alleles	N	Appendix table
BF-C2	866	A-1
B-BF	966	A-2
B-C2	897	A-3
B-DR	362	A-4
DR-BF	353	A-5
DR-C2	337	A-6
C4(complete)-C2	527	A-7
C4(complete)-BF	584	A-8
C4(complete)-B	592	A-9
C4(complete)-DR	255	A-10
C4(total)-BF	973	A-11
C4(total)-C2	905	A-12
C4(total)-DR	368	A-13
C4(total)-B	1036	A-14
C4-B-BF	960	A-15
C4-B-BF-C2	864	A-16
C4-B-BF-C2-DR	337	A-17

N = the number completely typed for the alleles shown and used for the appropriate association calculations.

a. Two-way associations between HLA-B, BF, C2, and HLA-DR alleles

Table IV-20 shows high positive non-random associations obtained by either the chi square method ($p<.05$) or by the delta standard method (%Ds$\geq$25). Uncorrected probability values are given in the table. Probability values scored with an asterisk are significant after correction for the number of comparisons made. Fifteen pairs are significantly associated by this latter criterion, including B12 and DRw7, Bw35 and DR1, BF*F and DR1, and BF*S and DR2. The strongest associations are between B8 and BF*S, B8 and DR3, B12 and BF*F, B7 and DR2, B17 and DR7, and Bw22 and C2*2.

Table IV-21 shows high negative non-random associations. In all cases strongly negative associations reflect positive associations, for example, B8 and BF*F, B7 and BF*F, etc.

b. Two-way associations between C4 haplotypes and HLA-B, BF, C2, and HLA-DR alleles

Associations between C4*A and C4*B alleles within C4 haplotypes have already been described (see Table IV-14). To calculate associations between C4 and the other MHC variants, the two C4 loci were treated as a unit, thus associations between C4 haplotypes and the other MHC alleles have been determined. Of the 1048 C4 haplotypes considered in this study, only 603 were "complete" haplo-

Table IV-20: Two-way positive associations between HLA-B, BF, C2, and HLA-DR alleles where number observed ⩾ 3 and either $p < .05$ or D+s ⩾ 25%.

HLA-B	BF	C2	HLA-DR	N	Nd. obs.	χ^2	p		D+ ($\times 10^4$)	D+s (%)
7	S			966	158	15.36	$.3 \times 10^{-5}$	*	278	87.17
8	S			966	135	30.71	$.2 \times 10^{-10}$	*	250	92.59
12	S1			966	4	2.92	.04		24	28.29
12	F			966	71	108.36	$.2 \times 10^{-20}$	*	470	36.24
17	S			966	25	1.73	.063		32	60.63
18	F1			966	5	67.26	$.1 \times 10^{-5}$	*	49	83.08
22	S			966	22	1.19	ns		27	57.67
27	S			966	33	1.18	ns		31	43.81
35	F			966	35	41.36	$.3 \times 10^{-8}$	*	220	32.52
37	F			966	5	3.42	.033		30	29.71
7		1		897	158	2.40	.036		47	58.80
8		1		897	120	5.31	$.3 \times 10^{-2}$		60	100.00
15		2		897	8	11.58	$.2 \times 10^{-2}$		62	14.73
22		2		897	13	145.22	$.1 \times 10^{-12}$	*	134	57.28
5			1	362	5	4.24	.024		86	19.29
5			4	362	8	8.62	$.3 \times 10^{-2}$		139	33.44
7			2	362	49	87.10	$.9 \times 10^{-18}$	*	861	53.62
8			3	362	45	159.13	$.1 \times 10^{-28}$	*	951	74.13
12			7	362	13	21.13	$.3 \times 10^{-4}$	*	242	31.75
14			7	362	9	6.29	$.7 \times 10^{-2}$		239	39.94
14			1	362	5	2.47	.049		74	13.60
15			4	362	10	14.64	$.3 \times 10^{-3}$		185	40.07
17			7	362	11	45.03	$.5 \times 10^{-7}$	*	250	69.47
18			2	362	5	1.36	ns		66	28.25
35			1	362	12	24.18	$.1 \times 10^{-4}$	*	238	30.10
40			4	362	7	4.51	.018		106	24.20
	F	1		866	149	6.82	$.8 \times 10^{-3}$	*	73	100.00
	S	2		866	37	7.59	$.4 \times 10^{-3}$	*	79	100.00
	F1	1		866	4	0.67	ns		2	100.00
	S1	1		866	8	0.08	ns		4	100.00
	S		2	353	72	6.48	$.2 \times 10^{-2}$		223	61.31
	S		3	353	56	3.14	.021		147	50.82
	S		4	353	61	4.10	.011		170	54.56
	S1		1	353	3	9.28	$.6 \times 10^{-2}$		63	41.89
	F		1	353	15	12.09	$.5 \times 10^{-3}$	*	234	22.21
	F		7	353	16	12.17	$.7 \times 10^{-4}$	*	271	26.88
		2	-	337	4	4.99	.020		83	21.96
		2	4	337	7	7.64	$.5 \times 10^{-2}$		131	38.83

*: Significant after correction for number of comparisons, see footnote, Table IV-21.

Table IV-21: Two-way negative associations between HLA-B, BF, C2, and HLA-DR alleles where number expected ≥ 3 and p < .05 or D-s ≥ 25%.

HLA-B	BF	C2	HLA-DR	N	No. obs.	χ^2	p		D- ($\times 10^4$)	D-s (%)
7	F			966	4	28.93	$.2 \times 10^{-9}$	*	-250	85.94
8	F			966	2	26.93	$.3 \times 10^{-9}$	*	-225	91.48
12	S			966	75	107.97	$.1 \times 10^{-20}$	*	-481	38.27
15	S			966	48	2.79	.027		-31	5.83
18	F			966	3	3.04	.023		-50	61.75
35	S			966	44	33.83	$.5 \times 10^{-7}$	*	-177	26.76
37	S			966	7	2.68	.045		-28	28.27
40	F			966	5	5.53	$.4 \times 10^{-2}$		-81	60.97
7		2		897	3	2.41	.036		-47	58.80
8		2		897	0	5.31	ns		-60	100.00
15		1		897	47	11.58	$.2 \times 10^{-2}$		-62	10.53
22		1		897	9	145.22	$.1 \times 10^{-2}$	*	-134	57.28
5			3	362	0	3.11	ns		-92	100.00
7			3	362	4	10.10	$.2 \times 10^{-2}$		-279	71.69
7			4	362	6	4.48	$.9 \times 10^{-2}$		-182	52.27
7			7	362	3	5.98	$.3 \times 10^{-2}$		-189	69.54
8			-	362	2	4.45	ns		-206	78.93
8			1	362	1	4.45	$.4 \times 10^{-2}$		-176	86.30
8			2	362	5	7.20	$.2 \times 10^{-2}$		-232	62.30
8			4	362	2	7.27	$.1 \times 10^{-2}$		-206	78.93
8			7	362	1	6.42	$.9 \times 10^{-3}$		-176	86.30
12			2	362	4	5.59	$.2 \times 10^{-2}$		-98	47.00
12			3	362	1	4.45	$.4 \times 10^{-2}$		-136	82.89
14			2	362	0	5.86	.026		-143	100.00
35			2	362	4	1.73	ns		-98	47.00
35			3	362	1	4.45	$.4 \times 10^{-2}$		-135	82.89
40			2	362	2	1.19	ns		-68	55.38
	F	2		866	0	6.82	$.8 \times 10^{-3}$	*	-4	100.00
	F1	2		866	0	0.67	ns		-2	100.00
	S1	2		866	0	0.08	ns		-4	100.00
	S		1	353	26	19.2	$.3 \times 10^{-4}$	*	-300	28.98
	S		7	353	25	17.45	$.6 \times 10^{-4}$	*	-283	28.57
	F		2	353	5	5.05	$.6 \times 10^{-2}$		-192	57.45
	F		3	353	4	3.57	ns		-151	57.26
	F		4	353	5	3.04	.023		-144	50.37

* Significant after correction for number of comparisons, i.e.

C2-DR: $p < .05/20 = .25 \times 10^{-2}$; C2-BF: $p < .05/8 = .01$

BF-DR: $p < .05/40 = .12 \times 10^{-2}$; C2-B: $p < .05/38 = .13 \times 10^{-2}$

B-DR: $p < .05/190 = .26 \times 10^{-3}$; BF-B: $p < .05/76 = .66 \times 10^{-3}$

types, definitely typed at both loci. In the remaining 445 haplotypes, a null allele could not be excluded at one or both loci. This typing problem, elaborated in Chapter IV, mainly involved AQOB1, A3B1, and AQOB1 haplotypes and the distortion of their frequencies in the totals. Associations between C4 haplotypes have therefore been calculated in two ways, (i) using the frequencies of "complete" haplotypes only (see Tables A-7 to A-10, Appendix A), and (ii) using the frequencies of the total "complete" and "incomplete" C4 haplotypes (Tables A-11 to A-14, Appendix A).

(i). Using the frequencies of "complete" C4 haplotypes only

High positive and negative associations obtained in this way are given in Tables IV-22 and IV-23, respectively. Twenty-two pairs have significant positive associations after probability values are corrected for the number of comparisons made, and the fairly close correspondence between chi square and delta standard methods is apparent. Negative associations reflect positive associations.

(ii). Using the frequencies of all "complete" and "incomplete" haplotypes

Non-random associations obtained using total C4-haplotypes for which appropriate typing data were available are given in Tables IV-24 and IV-25. Since the null allele could be excluded in most rare haplotypes, these figures are likely to accurately represent associations between rare C4 haplotypes and MHC alleles.

Table IV-22: Two-way positive associations between complete C4 haplotypes and other MHC alleles where number of C4 haplotypes observed ≥ 3 and either D+s ≥ 25% or p < .05.

C4	HLA -B	BF	C2	HLA -DR	N	No. obs.	χ^2	p	D+ ($\times 10^4$)	D+s (%)
AQOB1	8				592	92	351.5	$.9 \times 10^{-66}$*	1194	81.6
AQOB1		S			584	111	15.0	$.7 \times 10^{-5}$*	248	74.5
AQOB1			1		527	101	7.6	$.4 \times 10^{-3}$*	127	100.0
AQOB1				3	255	41	129.3	$.2 \times 10^{-25}$*	1184	73.3
AQOB2	40				592	7	57.3	$.2 \times 10^{-6}$ *	107	75.9
A2BQO	5				592	3	16.9	$.2 \times 10^{-2}$	46	47.9
A2BQO		F			584	3	9.2	.011	51	70.6
A2B1		S1			584	4	145.5	$.2 \times 10^{-6}$ *	67	65.6
A2B2	14				592	5	120.1	$.7 \times 10^{-7}$ *	81	61.4
A2B2				1	255	4	32.9	$.7 \times 10^{-4}$ *	144	78.7
A3BQO	12				592	36	55.5	$.1 \times 10^{-10}$*	392	32.1
A3BQO		F			584	33	40.7	$.3 \times 10^{-8}$ *	342	27.1
A3BQO		F1			584	5	22.4	$.7 \times 10^{-4}$ *	73	100.0
A3B1	7				592	71	54.6	$.9 \times 10^{-13}$*	549	55.1
A3B1			1		527	211	17.2	$.2 \times 10^{-5}$ *	230	85.9
A3B1				2	255	46	29.7	$.2 \times 10^{-7}$ *	748	52.9
A3B3	15				592	6	54.1	$.1 \times 10^{-5}$ *	93	73.1
A3B3				4	255	3	7.8	$.7 \times 10^{-2}$	95	100.0
A4B2	15				592	12	13.6	$.4 \times 10^{-3}$	128	23.2
A4B2	18				592	12	16.3	$.2 \times 10^{-4}$ *	134	26.6
A4B2		S			584	71	12.7	$.1 \times 10^{-4}$ *	190	91.8
A4B2			2		527	22	89.1	$.4 \times 10^{-14}$*	339	58.1
A4B2				4	255	14	11.8	$.5 \times 10^{-3}$	303	30.0
A4B4	22				592	12	289.3	$.2 \times 10^{-17}$*	195	79.6
A4B4			2		527	11	129.5	$.2 \times 10^{-12}$*	192	90.6
A4B4				-	255	3	6.7	.014	6	23.9
A6B1	17				592	18	279.2	$.2 \times 10^{-22}$*	286	70.8
A6B1				7	255	10	66.8	$.5 \times 10^{-9}$ *	345	89.8

* Significant after correction for number of comparisons, see footnote, Table IV-23.

Table IV-23: Two-way negative associations between complete C4 haplotypes and other MHC alleles where number of C4 haplotypes expected is ⩾ 3 and either D-s ⩾ 25% or p < .05.

C4	HLA -B	BF	C2	HLA -DR	N	No. obs.	χ^2	p	D- (x 10)	D-s (%)
AQOB1	7				592	3	19.1	.2 x 10^{-6}*	-273	84.2
AQOB1	12				592	3	15.3	.4 x 10^{-5}*	-233	82.0
AQOB1	35				592	0	8.4	.4 x 10^{-2}	-124	100.0
AQOB1		F			584	4	14.3	.9 x 10^{-5}*	-228	77.0
AQOB1			2		527	0	7.6	.8 x 10^{-2}	-127	100.0
AQOB1				2	255	2	13.9	.1 x 10^{-4}	-426	84.5
A2BQO		S			584	1	6.0	.8 x 10^{-2}	-39	70.0
A3BQO	8				592	2	16.7	.9 x 10^{-6}*	-240	87.6
A3BQO		S			584	49	50.7	.5 x 10^{-10}*	-401	32.3
A3B1	8				592	13	39.5	.9 x 10^{-11}*	-495	69.2
A3B1	17				592	2	9.3	.4 x 10^{-3}	-131	79.4
A3B1	22				592	0	10.3	.1 x 10^{-2}	-119	100.0
A3B1			2		527	2	17.2	.2 x 10^{-5}*	-230	85.8
A3B1					255	5	28.6	.5 x 10^{-8}*	-692	77.9
A4B2	8				592	1	14.0	.2 x 10^{-5}*	-202	92.2
A4B2		F			584	1	10.6	.3 x 10^{-4}*	-167	90.8
A4B2			1		527	40	89.1	.4 x 10^{-13}*	-339	30.9
A4B4			1		527	1	129.5	.1 x 10^{-12}*	-194	91.1

* Significant after correction for number of comparisons, i.e.
for C4 - HLA-B, $p < .2 \times 10^{-3}$
for C4 - BF, $p < .9 \times 10^{-3}$
for C4 - C2, $p < .2 \times 10^{-2}$
for C4 - HLA-DR, $p < .4 \times 10^{-3}$

Table IV-24: Two-way positive associations between complete and incomplete C4 haplotypes and other MHC alleles where number observed ≥ 3 and either D+s ≥ 25% or p < .05.

C4	HLA -B	BF	C2	HLA -DR	N	No. obs.	χ^2	p	D+ ($\times 10^4$)	D+s (%)
AQOB1	8				1036	92	468.1	$.4 \times 10^{-7}$ *	734	75.3
		S			973	111	18.1	$.6 \times 10^{-6}$ *	180	78.0
			1		905	101	4.4	$.6 \times 10^{-2}$	52	100.0
				3	368	41	154.2	$.8 \times 10^{-27}$*	868	74.4
	21				1036	4	2.7	.049	28	32.6
AQOB2	40				1036	7	54.6	$.3 \times 10^{-6}$ *	61	76.4
A2BQO	5				1036	3	15.5	$.3 \times 10^{-2}$	25	47.1
		F			973	3	5.6	.018	24	70.4
A2B1		S1			973	4	131.1	$.6 \times 10^{-6}$*	40	49.2
A2B2	14				1036	5	59.6	$.3 \times 10^{-5}$*	45	60.8
				1	368	4	19.5	$.5 \times 10^{-3}$	95	77.9
A3BQO	12				1036	36	48.8	$.2 \times 10^{-8}$*	302	41.7
	18				1036	11	10.8	$.1 \times 10^{-2}$	65	15.1
		F			973	33	25.4	$.1 \times 10^{-5}$*	181	24.6
		F1			973	5	32.4	$.3 \times 10^{-4}$*	45	80.5
A3B1	7				1036	71	36.6	$.3 \times 10^{-8}$*	300	22.5
			1		905	211	7.6	$.9 \times 10^{-3}$	86	78.9
				2	368	46	36.4	$.2 \times 10^{-8}$*	606	39.6
A3B3	15				1036	6	51.7	$.1 \times 10^{-5}$*	53	73.6
				4	368	3	8.6	$.6 \times 10^{-2}$	67	100.0
A4B2	15				1036	12	11.9	$.9 \times 10^{-3}$	72	11.8
	18				1036	12	22.2	$.4 \times 10^{-4}$*	84	19.0
		S			973	71	14.9	$.1 \times 10^{-5}$*	134	93.0
			2		905	26	185.9	$.1 \times 10^{-20}$*	253	58.9
				4	368	14	13.5	$.3 \times 10^{-3}$	222	31.1
A4B4	22				1036	12	356.4	$.8 \times 10^{-18}$*	113	79.5
			2		905	11	188.7	$.6 \times 10^{-14}$*	116	91.3
				-	368	3	6.6	.01	65	37.5
A6B1	17				1036	18	425.2	$.6 \times 10^{-25}$*	167	71.1
				7	368	10	56.6	$.4 \times 10^{-8}$ *	234	89.3

continued

Table IV-24: continued.

C4	HLA -B	BF	C2	HLA -DR	N	No. obs.	χ^2	p	D+ (10^4)	D+s (%)
A3A2BQO		F			973	3	8.9	$.5 \times 10^{-2}$	26	100.0
A3A2*BQO	35				1036	6	27.7	$.5 \times 10^{-4}$ *	50	56.0
		F			973	9	31.5	$.1 \times 10^{-5}$ *	74	87.0
				1	368	5	22.4	$.2 \times 10^{-3}$	116	68.3
A3A2*BQO	12				1036	4	11.2	$.3 \times 10^{-2}$	31	77.8
A3*A2B1*	35				1036	5	27.8	$.7 \times 10^{-4}$ *	42	67.8
		F			973	4	13.5	$.9 \times 10^{-3}$	34	100.0
A2B1*		S1			973	5	143.8	$.5 \times 10^{-7}$ *	49	44.5
A3*B3	15				1036	8	63.0	$.8 \times 10^{-7}$ *	69	64.1
A2B2B1*	14				1036	7	150.4	$.7 \times 10^{-10}$ *	65	100.0

* Significant after correction for numbers of comparisons made, i.e.
for C4 - HLA-B, $p < .1 \times 10^{-3}$,
for C4 - BF, $p < .4 \times 10^{-3}$,
for C4 - C2, $p < .8 \times 10^{-3}$, and
for C4 - HLA-DR, $p < .2 \times 10^{-3}$.

Table IV-25: Two-way negative associations between complete and incomplete C4 haplotypes and other MHC alleles where number expected ≥ 3, D-s $\geq 25\%$ and $p < .05$.

C4	HLA -B	BF	C2	HLA -DR	N^1	No. obs.	χ^2	p^2	D- ($\times 10^4$)	D-s (%)
AQOB1	7				1036	3	18.7	$.2 \times 10^{-7}$ *	-165	85.1
	12				1036	3	16.2	$.1 \times 10^{-5}$ *	-139	78.0
		F			973	4	17.2	$.6 \times 10^{-6}$ *	-170	80.6
				2	368	2	10.2	$.1 \times 10^{-3}$ *	-253	82.4
A3BQO	8				1036	2	12.7	$.1 \times 10^{-3}$	-98	83.8
		S			973	49	34.2	$.3 \times 10^{-7}$ *	-216	30.0
A3B1	8				1036	13	15.4	$.8 \times 10^{-5}$ *	-180	59.0
				3	368	5	8.6	$.5 \times 10^{-2}$	-378	73.6
A4B2	8				1036	1	8.6	$.1 \times 10^{-3}$	-83	89.3
		F			973	1	13.0	$.4 \times 10^{-5}$ *	-121	92.4
			1		905	40	185.9	$.1 \times 10^{-20}$ *	-253	36.4
A4B4			1		905	1	188.7	$.3 \times 10^{-14}$ *	-116	91.3
A3B1*	8				1036	0	16.2	$.1 \times 10^{-4}$ *	-130	100.0
A3*B1*	8				1036	9	11.4	$.8 \times 10^{-4}$ *	-138	61.5

* Significant after correction for number of comparisons. See footnote, Table IV-24.

For A3B1, AQOB1, and A3BQO haplotypes, comparisons are necessary between these results and those given in (1) above.

It can be seen that nineteen of the twenty-two significant pairs from the first set of results (Tables IV-22 and IV-23) are also significant in the second set (Tables IV-24 and IV-25). An additional seven pairs, involving "incomplete" haplotypes show positive non-random associations when the larger total is used.

c. Three-way associations among complement variants: C4 haplotypes and BF and C2

The distribution of C4 haplotypes by BF and C2 is shown in Table IV-26, and positive associations as judged by relative delta values are given in Table IV-27. Except for A3B1, A3*B1, and A3*B1*, haplotypes likely to be identical are grouped together in the latter table, and the calculations appear to be identifying up to 14 C4-BF-C2 combinations. A portion of the A3*B1 and A3*B1* haplotypes must be AQOB1, which may account for the higher relative delta values obtained for A3*B1 and A3*B1* combinations than for A3B1-S-1. Six combinations give relative deltas of 100%. These include the combinations containing both putative 'extra-gene' C4-haplotypes, A3A2BQO and A2B2B1, and the combinations containing AQOB2 and A3B3. Both C4-haplotypes containing A4 (A4B2 and A4B4) are associated

Table IV-26: Distribution of C4 haplotypes by C2 and BF.

C4	C2*1 or C2*1*				C2*2				Total
	BF*S	S1	F	F1	BF*S	S1	F	F1	
A3B1	172	0	35	0	2	0	0	0	209
A3B1*	54	0	25	0	1	0	0	0	80
A3*B1	66	0	9	0	0	0	0	0	75
A3*B1*	110	0	18	1	0	0	0	0	129
A3BQO	41	0	30	3	0	0	0	0	74
AQOB1	98	0	3	0	0	0	0	0	101
AQOB2	8	0	0	0	0	0	0	0	8
A2B2	8	0	1	0	0	0	0	0	9
A2B2*	1	0	0	0	0	0	0	0	1
A2B1	4	4	0	0	0	0	0	0	8
A2B1*	3	4	2	0	0	0	0	0	9
A2*B1	2	0	0	0	0	0	0	0	2
A2BQO	1	0	3	0	0	0	0	0	4
A3*B2	6	0	0	0	1	0	0	0	7
A2B1B2*	3	0	0	0	0	0	0	0	3
A2B2B1*	7	0	0	0	0	0	0	0	7
A3B2	2	0	0	0	0	0	0	0	2
A3*B2B1*	1	0	0	0	0	0	0	0	1
A3B3	6	0	0	0	0	0	0	0	6
A3B3*	1	0	1	0	0	0	0	0	2
A3*B3	7	0	1	0	0	0	0	0	8
AQOB3	0	0	1	0	0	0	0	0	1
A4B2	39	0	1	0	22	0	0	0	62
A4*B2*	1	0	0	0	0	0	0	0	1
A4B4	1	0	0	0	11	0	0	0	12
A6B1	20	0	1	0	0	0	0	0	21
A3A2BQO	0	0	3	0	0	0	0	0	3
A2*A3BQO	1	0	3	0	0	0	0	0	4
A3*A2BQO	0	0	8	0	0	0	0	0	8
A3*A2B1*	0	0	3	0	0	0	0	0	3
A3*A2B2*	0	0	1	0	0	0	0	0	1
Total	663	8	149	4	37	0	0	0	861

Table IV-27: Three-way positive associations between C4 haplotypes and the alleles of C2 and BF, where number observed ⩾ 3 and D+s ⩾ 25%.

C4	BF	C2	No.obs.	D+(x 10^4)	D+s(%)
A2B1B2*	S	1	3	9	100.0
A2B2B1*	S	1	7	21	100.0
A2B2	S	1	8	16	56.6
A3B1	S	1	172	209	32.7
A3*B1	S	1	66	124	54.2
A3*B1*	S	1	110	174	44.2
AQOB1	S	1	98	273	88.7
AQOB2	S	1	8	24	100.0
A3*B2	S	1	6	10	48.4
A6B1	S	1	20	52	81.3
A3B3	S	1	6	18	100.0
A3*B3	S	1	7	12	51.0
A2B1	S1	1	4	45	49.0
A2B1*	S1	1	4	45	49.0
A2BQO	F	1	3	27	71.3
A3BQO	F	1	30	206	27.7
A3A2BQO	F	1	3	29	100.0
A2*A3BQO	F	1	3	27	71.3
A3*A2BQO	F	1	8	78	100.0
A3BQO	F1	1	3	31	74.0
A4B2	S	2	22	254	59.4
A4B4	S	2	11	128	92.1

with BF*S C2*2.

d. Three-, four-, and five-way associations among C4 haplotypes and HLA-B, BF, C2, and HLA-DR .

The results from these calculations are summarized in Table IV-28. The relatively small amount of HLA-DR data has allowed identification of only nine five-component haplotypes, namely

HLA-B5	BF*S	C2*1	C4*A3B1	HLA-DR4
7	S	1*	A3B1	2
8	S	1*	AQOB1	3
15	S	1*	A3B3	4
15	S	2	A4B2	4
17	S	1*	A6B1	7
18	S	1*	A4B2	2
22	S	2	A4B4	-
35	F	1	A3*A2BQO	1

In addition, it can be seen from the four-way associations that a number of complement combinations identified in (c) above are associated with particular HLA-B antigens. These are

HLA-B12	BF*F	C2*1*	C4*A2*A3BQO
14	S	1*	A2B2
27	S	1*	A4B2
35	F	1*	A3*A2B1*
40	S	1*	AQOB2

The C4 haplotypes A2*A3BQO and A3*A2B1* are likely to be

Table IV-28: Three-, four-, and five-way positive associations among complete and incomplete C4 haplotypes, HLA-B, BF, C2, and HLA-DR alleles where D+s ≥ 25% and number of haplotypes observed ≥ 3.

HLA-B	C4	BF	C2(1)	HLA-DR	N	No. obs.	D+s (%)
5	A3B1	S	1	4	337	6	30.2
7	A3B1	S			960	68	28.3
7	A3B1	S	1		864	64	29.6
7	A3B1	S	1	2	337	32	43.3
8	AQOB1	S			960	91	88.8
8	AQOB1	S	1		864	80	76.2
8	AQOB1	S	1	3	337	35	75.6
12	A2*A3BQO	F			960	3	58.6
12	A2*A3BQO	F	1		864	3	73.2
14	A2B2	S			960	5	53.9
14	A2B2	S	1		864	5	54.5
14	A2B2B1*	S			960	7	100.0
14	A2B2B1*	S	1		864	7	100.0
15	A3B3	S			960	5	69.6
15	A3B3	S	1		864	5	70.1
15	A3B3	S	1	4	337	3	100.0
15	A3*B3	S			960	6	65.2
15	A3*B3	S	1		864	6	73.0
15	A4B2	S	2	4	337	4	28.7
17	A6B1	S			960	17	73.5
17	A6B1	S	1		864	14	69.2
17	A6B1	S	1	7	337	8	88.7
18	A3BQO	F1			864	5	82.5
18	A4B2	S	1	2	337	4	35.6

continued

Table IV-28: continued.

HLA-B	C4	BF	C2	HLA-DR	N	No. obs.	D+s (%)
22	A4B4	S			960	12	79.7
22	A4B4	S	2		864	10	83.5
22	A4B4	S	2	-	337	3	50.0
27	A4B2	S	1		864	8	24.9
35	A3*A2B1*	F			960	3	73.2
35	A3*A2B1*	F	1		864	3	100.0
35	A3*A2BQ0	F			960	6	60.2
35	A3*A2BQ0	F	1		864	6	72.4
35	A3*A2BQ0	F	1	1	337	4	57.2
40	AQOB2	S			960	7	76.1
40	AQOB2	S	1		864	6	73.6
40	A3*B2	S			960	3	25.5

(1) Includes C2*1 and C2*1*.

identical to each other and to the haplotypes A3*A2BQO and A3A2BQO. The C4 haplotypes A2B2 and A2B2B1* may also be identical. No A2B2 haplotype came from a family in which a B1 allele on the same chromosome could be definitely excluded, while all A2B2B1* haplotypes came from families in which the possession of two B genes on the same chromosome was the most likely interpretation.

C. C4 and other MHC allotypes in multiple sclerosis (MS) and insulin-dependent diabetes (IDDM) patients

1. A comparison of MHC allotypes in MS and non-MS haplotypes

Of the 366 founder or entry haplotypes collected from families with one or more cases of multiple sclerosis, 188 occurred in patients and 162 occurred in the patients' healthy relatives but not in the patients themselves. The former are referred to in the analyses which follow as MS haplotypes, the latter as non-MS haplotypes. Sixteen haplotypes could not be designated as MS or non-MS either because all members of a family were not haplotyped or because there was a homozygous parent and the two "identical" haplotypes could not be distinguished in the children. These sixteen haplotypes were omitted from the analyses which will be described.

Table IV-29 compares the distribution of HLA-B, BF,

Table: IV-29: Distribution of HLA-B, C4, BF, C2, and HLA-DR allotypes obtained by direct count from 188 founder haplotypes occurring in MS patients and from 162 founder haplotypes occurring in healthy members of the same MS families.

HLA-B	MS	non-MS	p
5	6	6	
7	49	25	.0054
8	27	22	
12	18	23	.0543
13	0	2	
14	7	11	
15	8	12	
16	2	8	
17	4	7	
18	10	5	
21	5	6	
22	4	5	
27	11	4	.0605
35	11	15	
37	6	0	.0237
40	16	5	.0187
42	3	1	
54	0	1	
-	1	1	
?	0	3	
	188	162	

BF	MS	non-MS	p
S	154	124	.0655
F	26	30	.0533
S1	2	2	
F1	0	2	
O?	1	0	
NT	5	4	
	188	162	

C2	MS	non-MS	p
1/1*	176	150	.0399
2	4	10	.0399
NT	8	2	
	188	162	

C4	MS	non-MS	p
A3B1	71	41	.0175
A3*B1	11	14	
A3B1*	9	16	
A3*B1*	14	10	
A3BQO	14	9	
AQOB1	25	21	
A4B2	16	18	
A6B1	4	4	
A3*A2BQO	5	1	
A2*A3BQO	1	0	
A3*A2B1*	0	2	
A2B2B1*	0	4	
A2B1B2*	0	2	.0327
A2B1	2	2	
A2B2	3	1	
A2BQO	1	2	
A2B1*	2	2	
A2B2*	0	1	
A3B2	1	1	
A3*B2	3	4	
AQOB2	2	0	
A3B3	1	4	
A2B3	1	0	
A4B4	2	3	
	188	162	

HLA-DR	MS	non-MS	p
1	13	6	
2	56	20	.000012
3	25	28	.0657
4	23	25	
5	10	8	
6	3	1	
7	13	18	.0554
8	4	1	
-	17	12	
?	10	18	
NT	8	2	
	188	162	

p = probability (Fisher's Exact Test)
NT = not tested

C2, and C4 allotypes in the MS and non-MS groups. The strongest association was observed between MS and HLA-DR2 ($p = 0.00012$), while HLA-B7 was also increased in the disease group ($p = 0.0054$) as, to a lesser extent, was the C4 haplotype A3B1 ($p = 0.0175$). The latter association was probably distorted somewhat by the existence of partial haplotypes, A3*B1, A3B1*, and A3*B1*, any or all of which could be A3B1.

There was also some perturbation in the distribution of other alleles, including a decrease of HLA-B16 in the patient haplotypes and a corresponding increase in the healthy group ($p = 0.0260$), an increase of B37 in the MS group ($p = 0.0237$), and likewise of B40 ($p = 0.0187$). Of the C4 haplotypes, only one other besides A3B1 showed an unusual distribution; putative double B haplotypes, A2B2B1* and A2B1B2*, were not observed in the patients ($p = 0.0327$). Finally, the proportion of C2*2 allotypes was lower in the group of MS-haplotypes than in the non-MS group ($p = 0.0399$).

2. The distribution of C4 alleles in MS and non-MS haplotypes

Table IV-30 shows the numbers of various individual C4 alleles in MS and non-MS haplotypes. A3 and B1 are increased in the MS group and these differences are significant. The B2B1 combination from putative "double B"

Table IV-30: Comparison of the distribution of C4 alleles obtained by direct count from 188 MS haplotypes to that obtained from 162 non-MS haplotypes.

C4 allele	MS haplotypes	Non-MS haplotypes	p
A3	96	71	.034
A3*	28	28	
AQO	27	21	
A4	18	21	
A2	9	14	
A6	4	4	
A3A2	6	3	
	188	162	
B1	113	82	.018
B1*	25	30	
B2	25	24	
B2*	0	1	
B3	2	4	
B4	2	3	
BQO	21	12	
B2B1	0	6	.032
	188	162	

p = probability (Fisher's Exact Test)

haplotypes was significantly decreased.

3. The proportion of the combination B7 C2*1 BF*S C4*A3B1 DR2 in MS and non-MS haplotypes

There were 316 haplotypes in the MS family collection for which typing data were complete for all five markers; 172 were MS haplotypes and 144 were non-MS. Because the individual allotypes HLA-B7, C4*A3B1, and HLA-DR2 are increased in the disease haplotypes, and because the combination B7 C2*1 BF*S C4*A3B1 DR2 is likely, by the criterion of relative delta (Table IV-16, p. 157), to be a linkage disequilibrium combination, the proportions of this combination in the MS and non-MS totals were compared. A3B1, A3*B1, A3B1*, and A3*B1* are combined in this comparison since, given the rarity of known AQOB1 and A3BQO on B7-S-DR2 haplotypes (1/41 and 2/41 respectively as compared with 38/41 A3B1), the incomplete haplotypes are likely to be A3B1. This particular MHC combination, B7-1-S-A3B1-DR2, constitutes approximately 17% of the MS haplotypes as compared to approximately 9% of the group of non-MS haplotypes ($p = 0.0045$).

Table IV-31 shows the proportions of B7-, A3B1-, and DR2-containing haplotypes in the 172 MS and 144 non-MS haplotypes which were typed for all markers. As before, (Table IV-29), all three haplotypes are increased in the MS group, DR2 being the most highly significant. The A3B1

Table IV-31: HLA-B7, C4*A3B1, and HLA-DR2 haplotypes occurring in MS and non-MS haplotypes typed for five MHC markers.

	MS	non-MS	p(1)
Total haplotypes typed for 5 markers	172	144	
Total B7 haplotypes	43	25	.0286
Total A3B1 haplotypes (2)	93	69	.0490
Total DR2 haplotypes	54	20	.000049
Total B7-S-1-A3B1(2)-DR2	30	11	.0045

(1) probability (Fisher's Exact Test)
(2) A3B1 + A3*B1 + A3B1* + A3*B1*

haplotypes used in this table include complete A3B1 and incomplete A3*B1, A3B1*, and A3*B1* haplotypes. Some of these, particularly non-B7 non-DR2 haplotypes, are likely to be A3BQ0 or AQ0B1, hence the difference between MS and non-MS is lower than that shown in Table IV-29, where incomplete haplotypes are separated.

Finally, the proportions of each of the total B7, A3B1, and DR2 haplotypes which were B7-S-1-A3B1-DR2 were calculated. Table IV-32 shows these results. Approximately 70% of the MS B7-haplotypes were S-1-A3B1-DR2 as compared to 44% of the non-MS B7's ($p = 0.047$). Thirty-two percent of the MS A3B1-haplotypes were B7-S-1-DR2 as compared to 16% of the non-MS A3B1's ($p = 0.0065$). Approximately equal proportions (55%) of MS and non-MS DR2-haplotypes were B7-S-1-A3B1. Similar calculations were not performed for BF and C2 since almost all B7-A3B1-DR2 haplotypes in both groups were BF*S and C2*1.

4. C4 haplotypes in IDDM families

Fourteen nuclear families with one case each of juvenile insulin-dependent diabetes were typed as part of the FS family collection, giving 55 haplotypes of which 28 occurred in IDDM patients. Table IV-33 shows the haplotypes which were observed in these families. No HLA-DR data were available. It can be seen that at least nine IDDM haplotypes as compared to, at most, two non-IDDM haplotypes are HLA-B8 and C4AQ0B1. Furthermore, four IDDM haplotypes

Table IV-32 : HLA-B7 BF*S C2*1 C4*A3B1 DR2 haplotypes expressed as a proportion of (i) total B7, (ii) total A3B1, and (iii) total DR2 in MS and non-MS haplotypes.

	MS	non-MS	
Total B7 haplotypes typed for five markers	43	25	
Total B7-S-1-A3B1(1)-DR2	30	11	
% of total	69.8	44.0	$\chi^2 = 2.204$ $p = .047$
Total A3B1(1) haplotypes typed for five markers	93	69	
Total B7-S-1-A3B1(1)-DR2	30	11	
% of total	32.3	15.9	$\chi^2 = 5.298$ $p = .0065$
Total DR2 haplotypes typed for five markers	54	20	
Total B7-S-1-A3B1(1)-DR2	30	11	
% of total	55.6	55.0	$\chi^2 = .023$ $p = .2047$

(1) A3B1 + A3*B1 + A3B1* + A3*B1*

Table IV-33: Twenty-eight haplotypes from 14 IDDM patients and 27 haplotypes found in healthy members of IDDM families.

IDDM				non-IDDM			
HLA-B	BF	C2	C4	HLA-B	BF	C2	C4
8	S	1*	AQ0B1	8	S	1*	A3B1
8	S	1*	AQ0B1	8	S	1*	A3B1
8	S	1*	AQ0B1	8	NT	NT	A3*B1
8	S	NT	A3*B1	8	S	1*	AQ0B1
8	NT	NT	AQ0B1				
8	S	1*	AQ0B1	7	F	1*	A3B1
8	S	1	AQ0B1	7	NT	NT	A3B1*
8	S	1*	AQ0B1	7	NT	NT	A3*B1*
8	S	1	AQ0B1	7	NT	NT	A3*B1*
8	S	1*	AQ0B1				
				12	NT	NT	A3*B1*
5	S	1*	A3BQ0	12	F	1*	A3*B1
5	S	1*	A3*B3	12	NT	NT	A3B1*
12	F	NT	A3*B1	15	F	1*	A3B1
12	NT	NT	A3B1*	15	NT	NT	A3B1*
12	S	1*	A3BQ0	15	S	1*	A6B1
12	S	1*	A3BQ0				
				17	S	1*	A6B1
15	NT	NT	A3*B3				
15	NT	NT	A3B3	18	NT	NT	A3B1
15	S	1*	A3B1	18	NT	NT	A3*B1*
17	S	2	A4B2	22	F	1*	A3B1
17	S	NT	A3B1	22	S	1*	A3*B1
27	NT	NT	A3B1*	27	S	1*	A3B1*
27	S	1*	A3B1				
				35	S	1*	A4B2
7	NT	NT	A3B1*	35	F	1*	A3B1*
40	S	1*	A3*B2	37	F	1*	A3B1
40	S	1*	A3B1				
40	S	1*	A3*B3	40	S	1*	A3*B1
40	NT	1*	A3*B1	40	S	NT	NT
				40	S	NT	A3*B1
				40	S	1	A3B1

NT = not tested

contain the rare variant C4B3. This variant did not occur in the non-IDDM haplotypes.

Because of the relatively small number of non-IDDM, control haplotypes in these families, a larger group of founder haplotypes was selected from the healthy members of the FS collection. In Table IV-34, the distribution of C4 haplotypes in the 14 IDDM patients is compared with the distribution of 98 C4 haplotypes of healthy, unrelated FS individuals. Two haplotypes, AQOB1, and A3B3 and A3*B3 combined, are increased in the patients, with relative risks of 5.2 and 16.2 respectively.

The true frequency of the AQOB1 haplotype is problematical in the control group since a large number of A3 and B1 alleles could not be definitely assigned (10 A3*B1, 9 A3B1*, and 32 A3*B1*). The B3 allele, on the other hand, is definite in all four haplotypes shown. This C4 variant is rare, occurring in 17 (1.7%) of 1031 non-IDDM haplotypes in the total family material used for this study.

The alleles HLA-B15, BF*S, C2*1, C4B3 are positively associated (Table IV-28); of 17 C4*B3 haplotypes from non-IDDM families, 11 have this haplotype and two have B15 with BF and C2 not tested. The remaining four are all C2*1 but one is B40 BF*S, one is B22 BF*S, one is B37 BF*F, and one is B18 BF*F. Of the four IDDM C4*B3 haplotypes, two are B15 BF*S C2-not tested, one is B40 BF*S C2*1, and one

Table IV-34: Distribution of C4 in 28 haplotypes from 14 unrelated juvenile insulin-dependent diabetes patients and in 98 haplotypes from 49 unrelated members of non-disease families. Haplotype frequencies are given in parentheses.

C4 haplotype	IDDM patients	Controls	Relative risk
AQOB1	8 (.2857)	7 (.0714)	(1)5.2
A3BQO	3 (.1071)	9 (.0918)	
A3B1	5 (.1786)	12 (.1224)	
A6B1	2 (.0714)	2 (.0204)	
A3*B3	3 (.1071)	1 (.0102)	(2)16.2
A3B3	1 (.0357)	0 (.0000)	
A4B2	1 (.0357)	8 (.0816)	
A3*B1	3 (.1071)	10 (.1020)	
A3B1*	1 (.0357)	9 (.0918)	
A3*B1*	0 (.0000)	32 (.3265)	
A3*B2	0 (.0000)	1 (.0102)	
AQOB2	0 (.0000)	1 (.0102)	
A3*A2B1	0 (.0000)	1 (.0102)	
A3*B2B1*	0 (.0000)	1 (.0102)	
A4B4	0 (.0000)	2 (.0204)	
A2B1*	1 (.0000)	1 (.0102)	
A2BQO	0 (.0000)	1 (.0102)	
Total	28 (1.000)	98 (1.000)	

(1) For AQOB1 and IDDM, χ^2 (with Yates' correction) = 7.60, p = .0004 (uncorrected) or p = .006 (corrected for number of comparisons = 17).

(2) For A3B3 + A3*B3 and IDDM, χ^2 (with Yates' correction) = 6.88, p = .008 (uncorrected) or p = .056 (corrected for number of comparisons = 7).

is B5 BF*S C2*1.

D. Quantitation of serum C4

1. Reliability of the method

Serum concentrations of C4 protein (mg%) were measured by single radial immunodiffusion (SRID) using commercial plates. To test the reliability of this procedure intra-class correlations were determined using C4 concentrations obtained from 19 freshly drawn and separated serum samples where each determination was duplicated on the same plate and from 31 fresh serum samples where each determination was duplicated on a different plate. The intra-class correlation coefficient (r_I) obtained for the intraplate comparison was 0.995 and for the interplate comparison was 0.933. Thus within and between plate errors can be taken as 0.5% and 6.7% respectively, which values are within the 15% allowable error suggested by the manufacturer.

2. Control panel

Fourteen volunteers from the laboratory staff were bled on the same day each week for six weeks and on the same day each month for the following four months. Minor illnesses reported by the volunteers were recorded. Samples were divided into three aliquots and two aliquots were stored at -70 °C. Serum C4 concentrations were determined on

a fresh aliquot of each sample on the day of bleeding. To test for the effect of storage, determinations were repeated on a set of samples stored at -70°C six months from the first day of bleeding, and on a second set of samples stored at -70°C nine months from the first sampling day. To test for the effect of handling, samples collected from the eleven panel members on a single sampling date (Jan 6) were retested after being thawed and frozen fifteen times, over a two-day period.

Figure IV-20 shows the complement profiles for the individuals sampled. For each individual, three sets of determinations are given, fresh, stored up to six months, and stored up to nine months. The C4 concentrations obtained from the samples which had been repeatedly thawed and frozen are given in Table IV-35.

Table IV-36 shows the mean C4 and standard deviation for the total sample of all individuals at each sampling date (up to 14 individuals/sample). Table IV-37 shows the mean C4 and standard deviation for each individual over the six-month sampling period (up to 11 samples/individual).

All individuals were not sampled on each day. Two dropped out of the group before the end (JR, LY). Five individuals were absent from work and missed a single sampling day. Four of these reported minor illnesses. Thus data were complete for seven individuals only. Table IV-38 shows the mean C4 concentrations and standard deviations

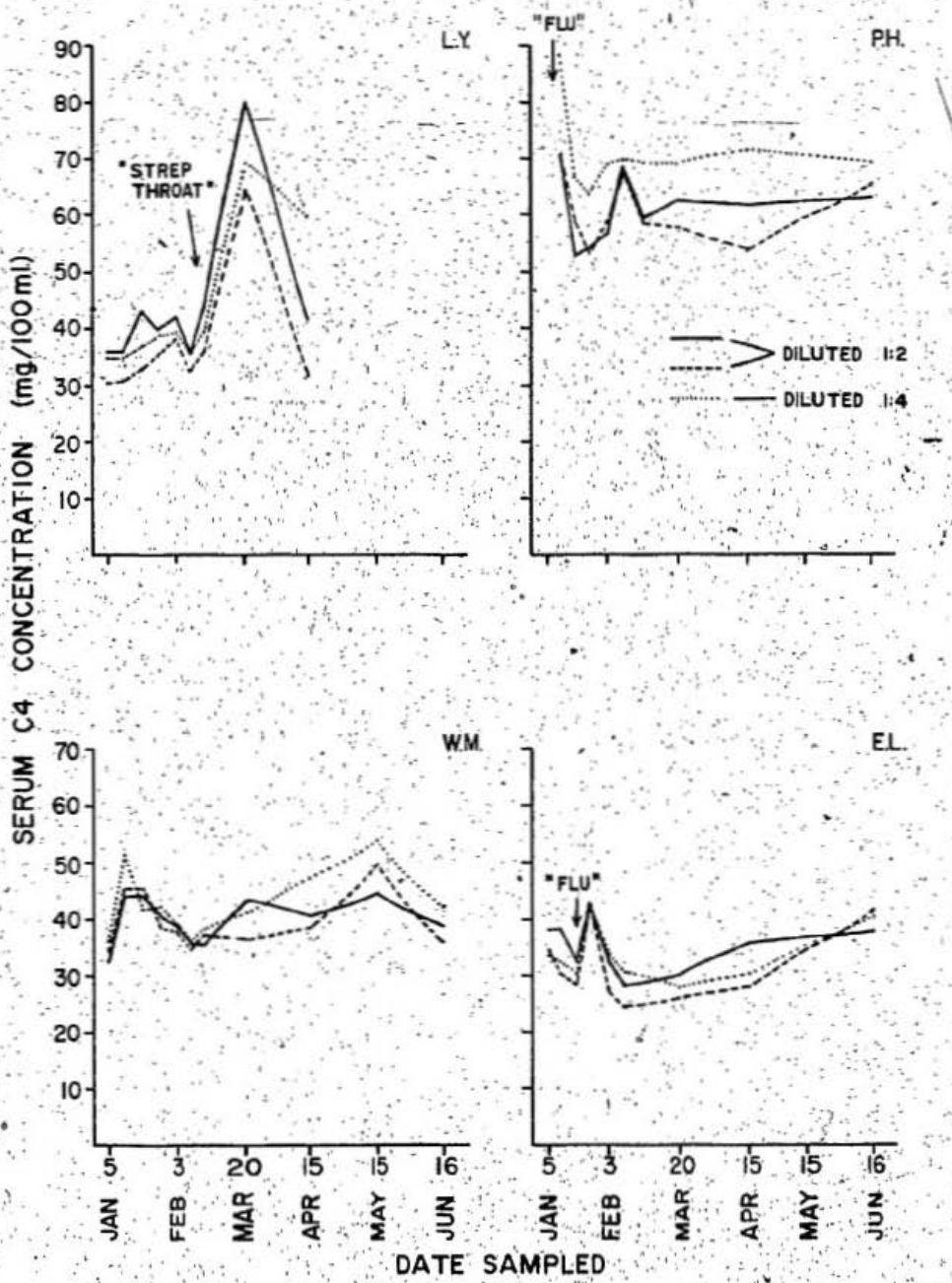


Figure IV-20: Complement profiles of 14 healthy volunteers sampled over a six-month period. (———) Samples tested fresh. (-------) Samples tested after storage at -70°C six months from the first sampling date. (——) Samples tested after storage at -70° C nine months from the first sampling date.

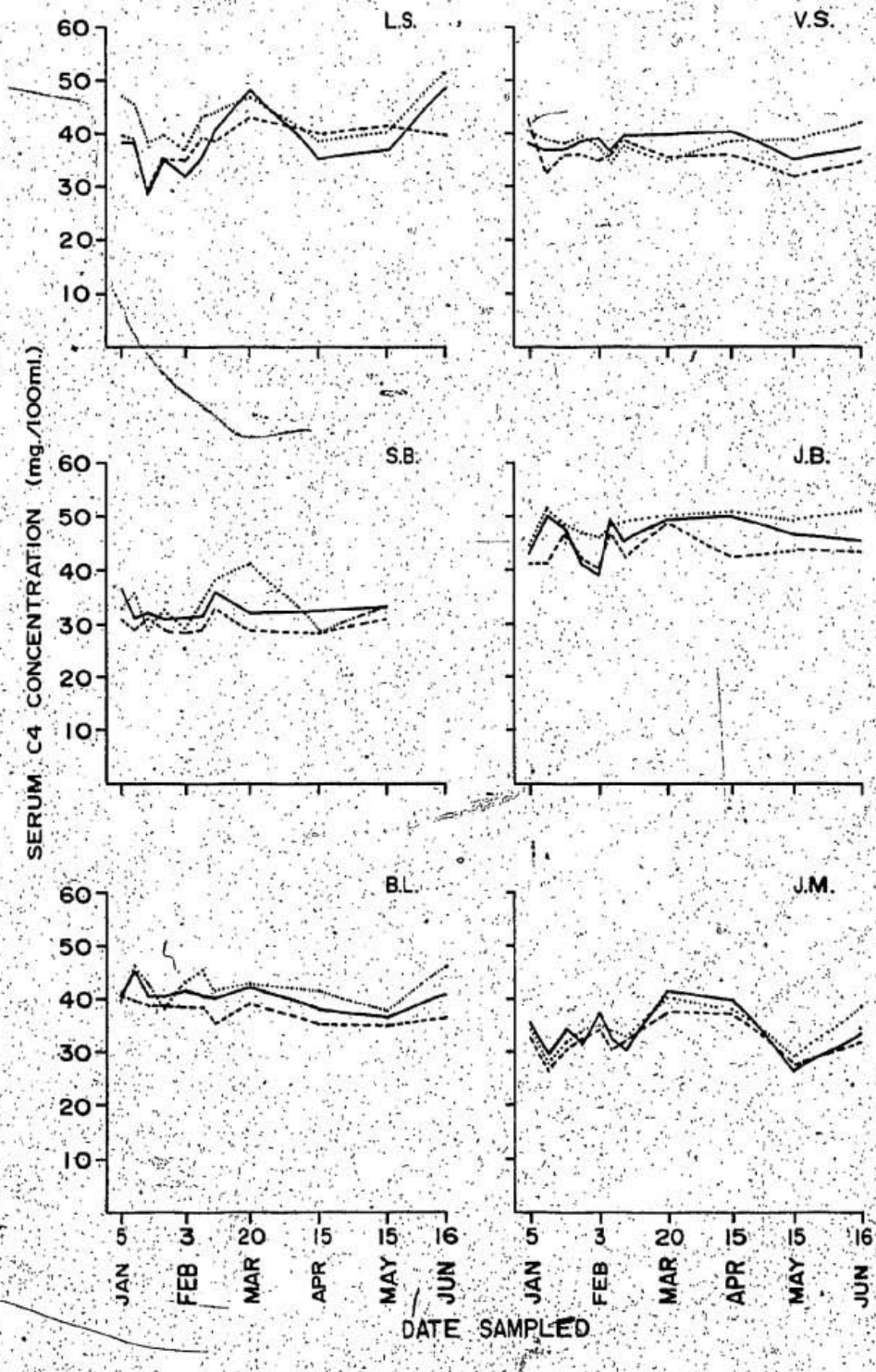


Figure IV-20: continued.

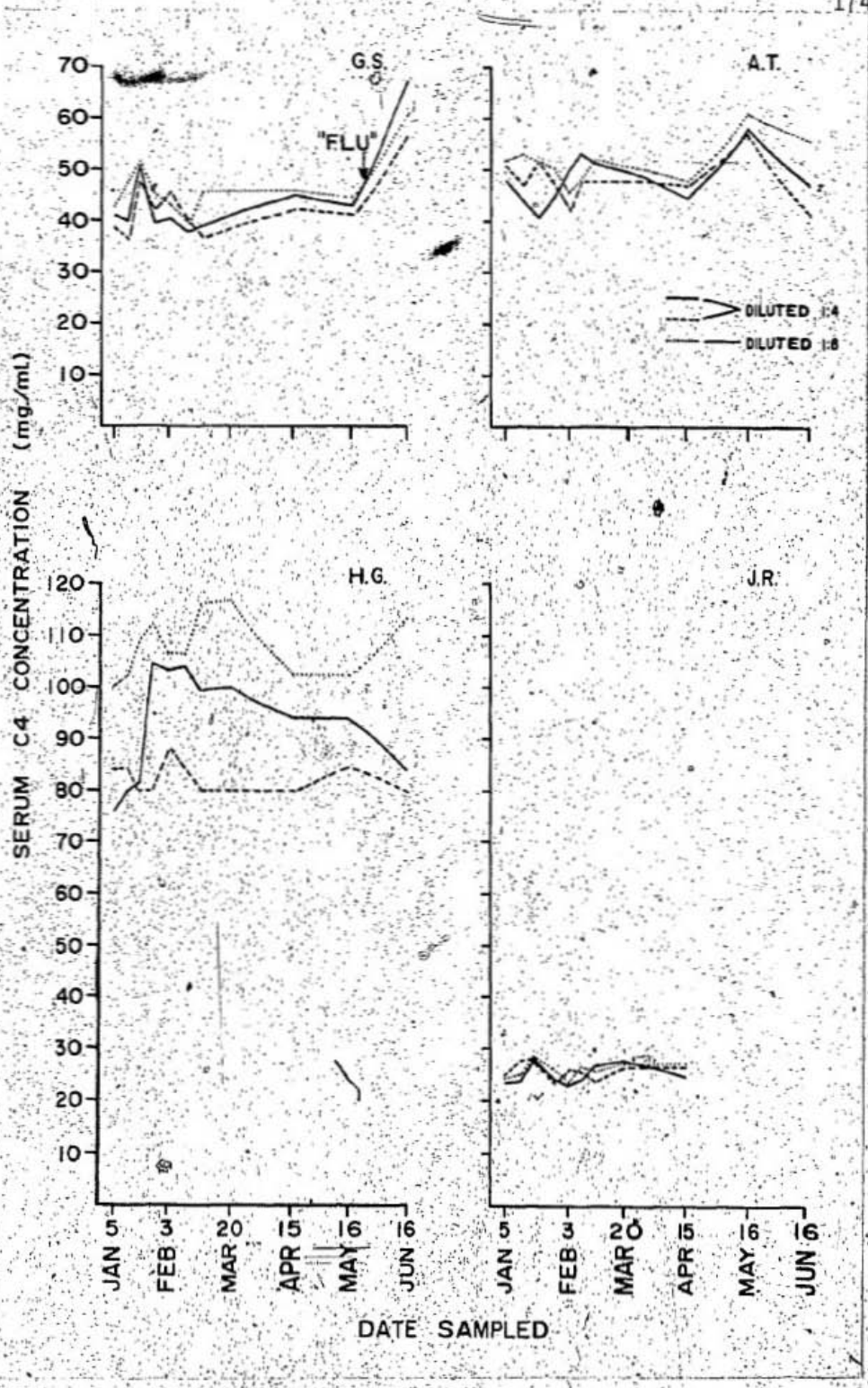


Figure IV-20: continued.

Table IV-35: C4 concentrations (mg%) of the same serum sample tested fresh and after being frozen and thawed 15 times.

Sample	Fresh	Frozen and thawed 15 times
EL	38.4	38.5
LS	38.4	39.6
VS	39.6	39.6
BL	40.8	38.4
JM	35.8	28.0
SB	36.4	39.6
JB	42.2	43.5
WM	32.4	32.8
GS	40.4	38.4
AT	47.6	49.6
JR	24.4	22.8

Table IV-36: Means and standard deviations of serum C4 concentration (mg%) for all individuals at each sampling date over a six-month period.

Date Sampled	N(1)	Fresh		6 months(2)		9 months(3)	
		$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
Jan 5	12	41.0	12.4	41.4	15.1	44.0	19.2
Jan 13	14	43.2	15.5	41.9	16.8	48.5	22.2
Jan 20	14	42.1	13.8	41.8	15.0	46.0	21.2
Jan 27	13	43.5	19.7	41.1	14.0	47.0	21.8
Feb 3	13(4)	42.7	14.7	42.4	15.5	45.3	20.6
Feb 10	14(5)	42.7	16.7	41.7	16.5	44.9	20.7
Feb 17	14	43.8	18.3	40.3	14.6	47.0	22.4
Mar 20	13	50.1	21.2	43.9	15.7	50.2	23.5
Apr 15	14	44.5	16.8	40.7	13.8	47.1	20.2
May 15	10	45.6	19.3	44.6	16.7	48.4	21.3
June 16	11	49.1	15.2	45.9	15.5	54.0	21.3

(1) Number of samples considered.
(2) Samples stored at -70 ° C 6 months from first sampling day.
(3) Samples stored at -70 ° C 9 months from first sampling day.
(4) N = 12 for fresh samples.
(5) N = 11 for fresh samples.

Table IV-37: Means and standard deviations of serum C4 concentrations (mg%) of all samples from each individual sampled over a six-month period.

Sample	N(1)	Fresh		6 months(2)		9 months(3)	
		$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
LY	8	45.8	16.2	38.0	10.9	44.5	13.0
EL	9	35.3	4.6	31.0	5.7	33.8	5.2
LS	11	37.8	6.0	37.8	4.0	42.7	4.4
VS	11	38.4	2.0	36,5	2.8	39.0	2.3
BL	11	40.4	2.4	37.7	1.9	41.9	2.8
JM	11	33.8	4.3	32.7	3.0	33.8	4.3
SB	10	31.9	2.2	29.5	1.8	32.2	4.7
JB	11	45.1	3.7	43.6	2.4	47.6	1.4
PH	9(4)	62.7	5.9	62.4	6.1	72.9	7.5
HG	11(5)	90.4	10.1	82.4	2.9	107.7	5.7
WM	11	40.1	4.4	39.6	5.0	41.6	6.0
GS	10	43.5	7.0	42.6	6.1	46.7	7.7
AT	11	48.1	4.9	47.8	4.3	51.2	4.3
JR	9	25.3	1.7	26.2	2.0	25.9	1.7

(1) Number of samples considered.
(2) Samples stored at -70°C 6 months from the first sampling day.
(3) Samples stored at -70°C 9 months from the first sampling day.
(4) 8 samples only for fresh samples.
(5) 9 samples only at 6 months.

Table IV-38: Serum C4 concentrations (mg%) in fresh and stored (-70°C) samples from seven individuals sampled over a six-month period.

Date tested	Sample name LS	VS	BL	JM	JB	WM	AT	$\bar{x}$
Jan 5 F	38.4	39.6	40.8	35.8	42.2	32.4	47.6	39.5
Jul -70°	39.6	43.2	40.8	32.8	45.2	34.4	50.0	40.9
Oct -70°	46.8	41.2	39.6	34.4	46.8	35.6	51.2	42.2
Jan 13 F	38.4	37.2	44.8	29.4	50.0	44.0	44.0	41.1
Jul -70°	38.4	32.8	39.6	28.0	45.2	45.2	46.8	39.4
Oct -70°	45.2	39.6	45.2	28.4	50.0	51.2	52.8	44.6
Jan 20 F	28.6	37.2	40.0	34.0	46.2	44.0	40.6	38.7
Jul -70°	28.4	36.8	38.4	30.4	46.8	45.2	51.2	39.6
Oct -70°	38.4	38.4	42.8	32.0	47.6	41.2	51.2	41.7
Jan 27 F	35.0	39.2	40.6	31.0	40.6	40.6	44.8	38.8
Jul -70°	34.4	36.8	38.4	32.0	41.6	38.4	46.8	38.3
Oct -70°	39.6	39.6	38.4	32.8	46.8	42.4	50.0	41.4
Feb 3 F	31.2	39.2	41.2	37.2	38.0	39.2	49.2	39.4
Jul -70°	34.4	35.6	38.4	34.4	39.6	38.4	42.8	37.7
Oct -70°	36.8	38.4	42.4	34.4	45.2	38.4	45.2	40.1
Feb 10 F	35.0	36.4	40.6	32.6	49.2	35.6	52.4	40.3
Jul -70°	38.4	36.8	38.4	35.6	45.2	34.4	47.6	39.5
Oct -70°	42.4	34.4	45.2	32.0	46.8	36.8	47.6	40.7
Feb 17 F	40.0	40.0	40.0	30.0	44.0	35.2	51.4	40.1
Jul -70°	38.4	38.4	35.6	32.0	41.6	36.8	47.6	38.6
Oct -70°	42.8	39.6	41.6	32.8	47.6	38.4	51.2	42.0
Mar 20 F	47.6	40.6	42.6	41.2	47.6	43.4	49.2	44.6
Jul -70°	42.8	35.6	38.4	36.8	46.8	36.8	47.6	40.7
Oct -70°	46.8	35.6	42.8	40.8	47.6	36.8	49.2	42.8
Apr 15 F	35.2	40.6	37.6	39.2	48.4	40.6	44.8	40.9
Jul -70°	39.6	36.8	35.6	36.8	41.6	39.6	46.8	39.5
Oct -70°	38.4	39.6	41.2	38.4	47.6	39.6	47.6	41.8
May 15 F	37.6	34.4	35.6	27.6	45.6	46.8	58.4	40.9
Jul -70°	41.2	32.8	34.4	28.8	42.8	50.0	57.6	41.1
Oct -70°	41.2	39.6	36.8	26.8	47.6	54.4	60.8	43.9
Jun 16 F	48.4	37.6	40.6	34.0	44.8	39.2	46.2	41.5
Jul -70°	39.6	35.6	36.8	32.0	42.8	36.8	41.2	37.8
Oct -70°	51.2	42.4	45.2	38.4	50.0	42.8	56.2	46.6
$\bar{x}$ F	37.8	38.4	40.4	33.8	45.1	40.1	48.1	40.5
$\bar{x}$ Jul -70°	37.8	36.5	37.7	32.7	43.6	39.6	47.8	39.6
$\bar{x}$ Oct -70°	42.7	39.0	41.9	33.8	47.6	41.6	51.2	42.5

obtained for the seven individuals at each sampling date, and for each individual over the six-month period or 11 sampling dates.

C4 phenotypes of the control panel are given in Table IV-39.

In order to examine the effect of storage on C4 concentration, each value obtained was scored according to weeks in the freezer (0 - 47 weeks) and an analysis of covariance by multiple regression was performed in which serum C4 concentration (mg%) was the dependent variable and date sampled and weeks in the freezer were independent variables. Sample values used in this analysis came from those seven individuals for whom the data were complete (Table IV-38). The results of the analysis are given in Table IV-40. Between-individual variation accounts for approximately 58% of the total variance (sum of squares/ total sum of squares x 100). Neither date sampled nor weeks frozen nor the interaction between these variables are statistically significant sources of variation. Weeks frozen accounts for 0.8% of total variance. Weeks frozen plus date sampled accounts for approximately 1.4%. Approximately 40% of the variance is residual, or not due to the variables tested here.

The following observations are made from these data:

(a). Individual variation

It can be seen from Figure IV-20 and from Tables IV-37

Table IV-39: Mean C4 concentrations and C4 phenotypes for 14 members of the control panel.

Sample	Mean serum C4 (mg%) $\bar{x}$ ± S.D.		C4 Phenotype
LY	45.8	16.2	A4A3B2
EL	35.3	4.6	A6A3B1
LS	37.7	6.0	A4B4B2
VS	39.0	2.0	A3B1
BL	40.4	2.4	A3B1
JM	33.8	4.3	A3A2B1
SB	31.9	2.2	A3B1
JB	45.2	3.7	A3B1
PH	62.7	5.9(1)	A6A3B3B1
HG	90.4	10.1(1)	A2B2B1
WM	40.1	4.4	A3B1
GS	43.5	7.0	A3B1
AT	48.1	4.9	A3B1
JR	25.3	1.7	A3B1

(1) estimate only (see text, p. 182)

Table IV-40. Analysis of covariance for individual C4 concentrations where the independent variables are date sampled and the number of weeks -70°C.

Source of variation	Sum of squares	df	Mean square	F	p
Between individuals	5098.01	6	849.67		
Within individuals	3656.49	224			
Date (D)	50.95	1	50.95	1.72	>.05
error	177.98	6	29.66		
Weeks frozen (W)	70.12	1	70.12	3.82	>.05
error	110.04	6	18.34		
D x W	1.14	1	1.14	<1	>.05
error	3246.26	209	15.53		
Total	8754.51	230			

df = degrees of freedom
p = probability

and IV-33 that 11 of the 14 individuals tested have C4 concentrations between 30-50 mg%. One individual was consistently lower (JR, x = 25.3 ± 1.7 mg%, fresh) and two individuals were consistently higher at 60-70 mg% (PH) and 90-110 mg% (HG). These latter individuals gave technical problems. Using 1:2 dilutions for these samples, their C4 levels were above the range of accuracy of the reference curve. Concentration values given for these samples, determined fresh and in July (6 months) are therefore estimates only. For the final determinations (October, 9 months) HG was diluted 1:8 and PH 1:4, which gave readings within the accuracy range of the reference curve. The October curves are therefore likely to be accurate.

(b). Within-individual vs between-individual variation

The analysis of variance results in Table IV-40 indicate that variation between individuals accounts for 58% of the total variance, as compared to within-individual variation, given in this analysis as sampling date, which accounts for only 0.5%. The seven individuals included in this analysis, however, did not report illness during the sampling period.

Table IV-37 shows the mean C4 concentration for each panel member over the entire sampling period. Table IV-36 shows the mean C4 concentation of all individuals tested for each sampling date. Standard deviations for the former range from approximately 4 - 16% of means (except LY at

approximately 30%). Standard deviations for the latter range from approximately 30 - 45% of means. Thus the variation in C4 levels in a single individual over time is still markedly less than variation among individuals when all individuals, including those reporting illness during the sampling period, are considered.

(c). Effect of illness

Four people missed their pre-designated sampling because of illness. These illnesses are indicated in Figure IV-20. Three individuals reported "flu" attacks. In each of these (PH, GS, EL) there is a moderate elevation in C4 levels on the next sampling date. One individual, (LY) reported "strep throat" which was confirmed by a physician and by culture. This individual showed an approximately two-fold increase of serum C4 on the next sampling date.

(d). Effect of handling

Values obtained from stored samples which had been thawed and frozen 15 times were not significantly different from those obtained from fresh or stored only samples (Table IV-35).

(e). Effect of storage

Length of storage accounts for a relatively small proportion of the variation seen in the samples, as indicated by the data presented in Tables IV-38 and IV-40. Differences are generally small and within the error of the method (taken as a maximum of 15%). The values obtained in

the last set of readings are generally somewhat higher than those from obtained from samples tested fresh and in July but this difference is unlikely to be an effect of storage since, for example, an individual sample taken in June and tested in October was stored approximately 16 weeks, whereas a sample taken in January and tested in July was stored approximately 26 weeks. The slightly higher October readings are more probably due to a small systematic error introduced into the testing procedure by the necessity of using a new batch of standard sera for the October readings. This error is likely to account for a proportion of the residual variance indicated in Table IV-40.

3. The effect of age, sex, and/or multiple sclerosis on serum C4 concentration

The technique of radial immunodiffusion was used to measure serum C4 concentrations of all samples from the MS family collection stored at -70°C, and these data were used to assess the effect of age, sex, and the disease on levels of serum C4. From the total tested, 122 completely unrelated individuals, i.e. possessing two entry MHC-haplotypes, were selected. This group contained 53 males and 69 females. The mean age of the group was 58.5 $\pm$ 11.6 years, and 21 individuals had multiple sclerosis. The mean C4 concentration was 45.9 $\pm$ 13.9 mg%.

Table IV-41 summarizes the results of an analysis of

Table IV-41: Analysis of covariance for serum C4 concentration (mg%) using the variables age, sex, and disease status, in 122 completely unrelated individuals from the MS family material.

Source of variation	Sum of squares	df	Mean square	F	p
Age (A)	1.17	1	1.17	0.01	n.s.
Sex (S)	12.39	1	12.39	0.06	n.s.
Disease status (D)	225.01	1	225.01	1.14	n.s.
A x S	79.00	1	79.00	0.40	n.s.
A x D	117.32	1	117.32	0.60	n.s.
S x D	193.98	1	193.98	0.99	n.s.
A x S x D	124.57	1	124.57	0.63	n.s.
Error	22451.48	114	196.94		
Total	23371.38	121			

Disease status (D) = with or without MS
n.s. = not significant

covariance by multiple regression using serum C4 (mg%) as the dependent variable, and the independent variables age, sex, and disease status. It can be seen that no variable alone, nor any interaction among variables, significantly affects the level of serum C4.

When the analysis was repeated using a larger sample (N=195) containing an additional 73 individuals with one entry haplotype, i.e. sharing one haplotype with one other individual in the group, the same result was obtained (Table IV-42). There was no effect of age, sex, or disease on C4 concentration.

4. The effect of number of C4 genes on serum C4 concentration

Forty of the 122 unrelated individuals considered above had C4 haplotypes from which a null allele could not be excluded at one or both loci. The remaining 82 possessed two completely-typed C4 haplotypes. The genotypes of this latter group were used to categorize them as two-, three-, or four-gene individuals in the following manner:

two-gene individuals = AQOB1/A3BQO, AQOB1/AQOB2, etc.

three-gene individuals = A3B1/AQOB1, A4B2/A3BQO, etc.

four-gene individuals = A3B1/A3B1, A4B2/A6B1, etc.

Of the 82 individuals considered, eight had two C4-genes, 42 had three C4-genes, and 32 had four C4-genes. There were 37 males and 45 females. The mean age was 57.4 $\pm$ 11.9 years

Table IV-42: Analysis of covariance for serum C4 concentration (mg%) using the variables age, sex, and disease status, for 195 unrelated and partially related (1) individuals from the MS family material.

Source of variation	Sum of squares	df	Mean square	F	p
Age (A)	277.26	1	277.26	1.38	n.s.
Sex (S)	3.18	1	3.18	0.02	n.s.
Disease status (D)	138.43	1	138.43	0.69	n.s.
A x S	285.62	1	285.62	1.42	n.s.
A x D	402.57	1	402.57	2.00	n.s.
S x D	413.70	1	413.70	2.06	n.s.
A x S x D	128.88	1	28.88	0.64	n.s.
Error	37577.11	187	200.95		
Total	39779.29	194			

Disease status = with or without MS
n.s. = not significant

(1) 73 individuals with one entry haplotype, i.e. share one haplotype with one other individual in the sample, and 122 with two entry haplotypes.

and the mean C4 concentration was 45.2 ± 14.4 mg%.

The results of a multiple regression analysis using serum C4 concentration as the dependent variable and including number of C4 genes as an independent variable, are given in Table IV-43. Number of genes alone is the only significant source of variation ($F = 13.9$, $p < .001$) accounting for 15.9% of the total variance (sum of squares/total sum of squares x 100).

When the analysis was repeated using a larger sample (N=127) which included an additional 45 individuals with one entry haplotype only, i.e. sharing one haplotype with one other individual in the group, the same result was obtained (Table IV-44). Of the variables tested, only the number of C4 genes significantly affects the level of serum C4.

5. Comparison of serum C4 levels in MHC-identical and MHC non-identical siblings

If serum C4 concentration reflects, to some degree, the number of C4 genes an individual possesses, then the levels of serum C4 in C4-identical (and MHC-identical) siblings should be more similar than in C4-different (and MHC-different) siblings. To test this hypothesis, two groups of sibling pairs were selected from the MS families. Group I contained 192 sib-pairs, the members of each pair sharing both MHC haplotypes (HLA-A, -B, -DR, and C2, C4,

Table IV-43: Analysis of covariance table for serum C4 concentration (mg%) using the variables age, sex, disease status, and number of C4 genes, in 82 competely unrelated individuals from the MS family material.

Source of variation	Sum of squares	df	Mean square	F	p
Age (A)	23.53	1	23.53	0.12	n.s.
Sex (S)	0.50	1	0.50	0.00	n.s.
Disease status (D)	399.05	1	399.05	2.09	n.s.
No. of genes (G)	2651.32	1	2651.32	13.90	<.001
A x S	185.26	1	185.26	0.97	n.s.
A x D	392.37	1	392.37	2.06	n.s.
A x G	40.72	1	40.72	0.21	n.s.
S x D	6.68	1	6.68	0.04	n.s.
S x G	21.53	1	21.53	0.11	n.s.
D x G	260.19	1	260.19	1.36	n.s.
A x S x D	105.81	1	105.81	0.55	n.s.
A x S x G	0.00	1	0.00	0.00	
A x D x G	0.00	1	0.00	0.00	
S x D x G	43.23	1	43.23	0.23	n.s.
A x S x D x G	0.00	1	0.00	0.00	
Error	12588.69	66	190.74		
Total	16689.68	81			

Disease status (D) = with or without MS
n.s. = not significant

Table IV-44: Analysis of covariance for serum C4 concentration (mg%) using the variables age, sex, disease status, and number of genes, of 127 unrelated and partially related(1) individuals from the MS family material.

Source of variation	Sum of squares	df	Mean square	F	p
Age (A)	15.26	1	15.26	0.07	n.s.
Sex (S)	4.55	1	4.55	0.02	n.s.
Disease status (D)	147.74	1	147.74	0.75	n.s.
No. of genes (G)	3669.37	1	3669.37	18.70	<.001
A x S	60.22	1	60.22	0.31	n.s.
A x D	519.49	1	519.49	2.65	n.s.
A x G	5.35	1	5.35	0.03	n.s.
S x D	10.71	1	10.71	0.05	n.s.
S x G	44.43	1	44.43	0.23	n.s.
D x G	253.19	1	253.19	1.29	n.s.
A x S x D	135.69	1	135.69	0.69	n.s.
A x S x G	0.00	1	0.00	0.00	
A x D x G	39.34	1	39.34	0.20	n.s.
S x D x G	148.27	1	148.27	0.76	n.s.
A x S x D x G	0.00	1	0.00	0.00	
Error	21783.64	111	196.25		
Total	26764.19	126			

Disease status = with or without MS
n.s. = not significant

(1) 45 individuals with one entry haplotype, i.e. share one haplotype with one other individual in the sample, and 82 with two entry haplotypes.

and BF). Group II contained 178 sib-pairs, the members of each pair being completely different for both haplotypes. In other words, in Group I sibs inherited the same two haplotypes from their parents whereas Group II pairs inherited two different ones. For each family considered, all possible combinations of siblings were used. For each pair, the difference in C4 concentration was obtained by subtraction. Table IV-45 shows the mean difference in C4 concentration for the two groups.

The difference in C4 level is clearly highly variable for both MHC-identical and non-identical siblings, and the mean difference in C4 concentration is similar for both groups of sibling pairs.

Table IV-45: Mean differences in serum C4 concentration (mg%) for MHC-identical and MHC non-identical sibling pairs from the MS families.

Group	No. of pairs considered	Mean difference in C4 conc.(mg%)
I (share both haplotypes)	192	13.45 ± 10.58
II (share neither haplotype)	178	14.58 ± 11.82

CHAPTER V DISCUSSION

A. Allotypes

1. Untreated plasma samples

Neuraminidase-treated plasma is superior to native plasma for separating C4 variants as can be seen from the data in this thesis. For example, only six phenotypes were clearly distinguished in 241 founder haplotypes derived from typing untreated samples. Of these, 108 were C4 FS with no A4 equivalent being observed. Only one haplotype with the I (A2) allotype and four with M* (B2) were observed. When these numbers are compared with the proportions of individuals found with A4, A2, and B2 using neuraminidase-treated samples (Tables IV-10 and IV-11, pp. 126, 127), the obvious conclusion is that many were missed. Finally, no B3 or B4 equivalents were observed when native plasma was used, and a number of samples could not be phenotyped at all because of heavy, blurred precipitates in the intermediate regions of the electrophoretic pattern.

Two patterns are of particular interest. There were seven C4*DS haplotypes containing the D (fast F) variant. The observations that (a) five of these (two were not tested) were functionally inactive in the D region and (b) five of the seven haplotypes were also HLA-B17, confirm earlier findings by, for example, Olaisen et al. (1980b) of

a functionally-inactive variant associated with HLA-B17. The DS haplotype was renamed A6B1 after neuraminidase treatment.

One variant, found on the haplotype C4*FSx only, was called Sx because, while the whole pattern appeared to be electrophoretically F, it was Rg+Ch+. This haplotype was subsequently shown to be A3B3.

2. Neuraminidase-treated samples

a. Electrophoretic patterns

In my typing of 1857 samples after neuraminidase treatment, I have recognized five A and five B (including AQO and BQO) patterns. There has been occasion to compare these patterns with others since, in 1981, ten laboratories, including this one, participated in a C4 reference typing of seventeen plasma samples provided by Dr. G. Mauff of Cologne. The results indicated clearly that all participants using neuraminidase-treated samples and the A-B nomenclature were identifying the same electrophoretic patterns as A6, A4, A3, some A2, B2, and B1. Results for the rarer, and mostly intermediate, patterns, were less clear.

Patterns called A2 in this study may not all be exactly the same. A2 on some A3A2 haplotypes seems closer to A3 than does A2 on some A2B1, A2BQO, and A2B1* haplo-

types. Five samples in the 1981 reference series were typed A2 by me and by at least three other participants. Of these, two were named Bn (for new B variant) by the co-ordinating laboratory. The electrophoretic pattern of these two variants was identical to the pattern observed by me in phenotypes which contain the A3A2BQ0 haplotype. If there are really two different allotypes, one on the A3A2BQ0 haplotype and the other A2 alone, then the distance between them is so small that in most runs a distinction cannot be made.

Comparison of the A2, B3, and B4 reported here with the allotypes shown in Figure I-4 (p. 37) indicates that the pattern called A2 in this study is likely to be identical to the allotype called A2 by the authors of Figure I-4. B3 is B3Bt (and B4Col), and B4 is B6Bt (and B5Col). Furthermore, the B3 and B4 allotypes are also likely to be identical to the ones called S4 and S5 respectively by Bruun-Petersen et al. (1981).

One other variant has been observed by me. This is an A variant, called A5, which is intermediate in position to A6 and A4. It was observed in a single family from whom samples were received after the study presented here was completed. The A5 pattern is very distinct and easily recognizable, i.e. has not been missed in my previous typings.

Where precipitin bands are very close together,

distinguishing them depends to a large extent on the type of agarose used, that is, on the property of endoosmosis. The agarose used in this study seemed to give the best compromise between sharp definition of the bands and distance between them. A few rare variants, particularly in the B region, may have been missed. It is just as likely, however that they simply did not exist in the populations studied.

It is interesting to note that although the allotype A4 occurs fairly frequently (8% in this study, 6,5% by Raum et al., 1980), there is only one report of a possible A4 equivalent by an investigator using native plasma. Teisberg et al. (1980b) have described a haplotype D2M which appears to be identical to A4B2. Bruun-Petersen et al. (1981), in a detailed report of C4 patterns seen after desialating plasmas, have included A4 (called F4) in a schematic diagram of gene products (Figure I-5b, p. 39) but they observed this allotype only in their population material and in individuals selected for the MHC haplotype A25 B18 C2*QO. While A4 is usually missed when native plasma is used, it can readily be distinguished after neuraminidase treatment. A4/A4 or A4/AQO genotypes are clearly seen and A4/A3 genotypes are less clear, but sufficiently different from A3 alone to be noted. Although 12/34 B18 haplotypes observed in this study were C4*A4B2 and a single confirmed B18 C2*QO was also A4B2, the A4 allotype was observed 74 times on a variety of other haplotypes, notably as A4B2 on

B15 C2*2, on B27 C2*1, and on B40 C2*1, and as A4B4 on B22 C2*2. This raises the possibility that the Danish and North Amerindan populations genuinely differ with respect to the distribution of this allele.

b. Duplicated A and B loci

A number of possible "double" haplotypes, that is, having two A or two B genes per haplotype, have been observed here. Three are definite A3A2BQO haplotypes and four are probable A2B2B1 haplotypes. In addition, there are a further 23 haplotypes for which the A3A2 interpretation seems to best fit the electrophoretic and segregation patterns and six for which B2B1 is the best interpretation.

Similar haplotypes have been reported by others. Nordhagen et al. (1981) have described FI (A3A2) haplotypes. Ramaika et al. (1982) have described one A3A2BQO haplotype and two with A2B2B1. Bruun-Petersen et al. (1982) have reported a single family with two haplotypes F3 F2.2 and S5.1 S1. The former is likely to be identical to A3A2, and the latter contains a variant S5 which has faster mobility than the B2 of B2B1.

In none of these cases, including the ones reported here, have two separate molecular populations been demonstrated or, put another way, has it clearly been shown that an individual with the genotype A3A2BQO/AQOB1, for example, possesses two discrete populations of A molecules.

Both A3 and A2 are Ch-Rg+ or Ch-Rg(part) and cannot presently be separated with antisera to Ch and Rg antigens. The possibility that the precipitin pattern represents a single gene product with an electrophoretic position similar to A3A2 cannot be excluded. The same interpretation can be applied to the B2B1 portion of the A2B2B1 haplotype.

Most double B haplotypes reported so far appear to contain three alleles, being A2B2B1, or A2B9B1 (Ramaika et al., 1982). Most double A haplotypes, including those reported here, have no demonstrable B alleles and are A3A2BQO (or A3A2B1*). This means that one of the so-called A patterns in these haplotypes, most likely A2, could be an unusual B gene product which is functionally weak and Ch-. The distinction between A and B genes by the possession of Rg and Ch antigens is not absolute, as can be seen from Figure IV-11, (p. 111), and will be discussed later. In this respect a single A2B2B1 haplotype reported by Hauptmann et al. (1982), the A2B9B1 reported by Ramaika et al. (1982), and the putative A2B2B1 haplotypes reported here are particularly interesting since they definitely appear to contain three genes.

Approximately 25% of the haplotypes observed in this study are A3BQO, AQOB1, or some other "one-gene" variety. A small number (up to 3%) are "two-gene" of the type A3A2BQO or "three-gene" A2B2B1 haplotypes. The remainder, approx-

imately 70%, are the usual "two-gene" haplotypes, A3B1 being the most common. Many families, however, have the phenotype A3B1 only, that is have A3*B1* haplotypes and are in no way informative. Any of these haplotypes could be A3A3B1 or A3B1B1 or some other "multi-gene" combination. The possible existence of these gives new meaning to the suggestion by Olaisen et al. (1979) that the number of genes an individual possesses is in itself a polymorphism, and raises some interesting new problems, particularly with respect to interpreting individual variation in serum levels of C4 protein.

c. Rodgers and Chido antigens

A6, A4, most A3, and A2 gene products, where typed and informative, have been shown here to carry the Rodgers antigen. Similarly I have found B2 and B1 to carry Chido. Some rare allotypes, however, have unusual Rodgers or Chido antigens. Three definite A3A2BQO haplotype products and one probable one (A3*A2B2*) were found to be Ch- Rg(part). This is in agreement with Nordhagen et al. (1981) who have described Rg(part) C4*FI products. These investigators have also reported Rg(part) C4*I products. In this study A2B1, A2B2, and A2B2B1* haplotypes, where tested, were found to be Rg+. Furthermore, nine A3BQO haplotypes were found to be Rg(part).

Nordhagen et al. (1980) have identified Chido partial inhibitors and in all cases this trait, partial inhibition,

was associated with the haplotype C4*M. Bruun-Petersen and Lamm (1980) have identified a haplotype product "G" which is a weak Chido inhibitor. In this study, samples were Chido-typed before C4 typing was performed, and the phenomenon of Chido partial inhibition was not anticipated. All informative B2 (or M) products tested were found to be Ch+, but three informative B3 products gave weak inhibition. B3 appears to be similar in electrophoretic position to the "G" variant of Bruun-Petersen and Lamm but comparison is difficult since the latter variant was observed using native plasma. Furthermore, although it is certainly different from B2 (M) when neuraminidase-treated plasma is used, B3 cannot be readily distinguished from the product M in native plasma.

Both B3 and B4 gene products show considerable variability with regard to Chido antigens (see Tables IV-5 and IV-6, pp. 108, 109) which is difficult to explain. Although it seems unlikely, it may be that all B3 and B4 products are Chido partials and samples typed positive and negative are actually Chido partial inhibitors which have been misclassified. As was stated above, the phenomenon of partial inhibition was not a result anticipated at the time of typing. Alternatively, and more likely, Chido determinants, whether present, absent, or "partial", bestow another level of variability on these seemingly electrophoretically identical molecules. In other words, all molecules classified B3 or B4 with the present technique may not be the

same. Only in informative families, and assuming one A and one B locus per haplotype, can these variants be assigned to the A or B locus. Naming uninformative ones is a problem.

The family illustrated in Figure IV-11 (p. 111) is a case in point. Five of the six family members are informative for Chido antigen. As was expected, individuals with the genotype A3BQO/A3BQO were Ch-. Two individuals have the genotype A3BQO/A2B3 and were also Ch-. The variant was classified B3 by its position and by its strong haemolytic activity after neuraminidase treatment. Two interpretations are possible. Either the haplotype A2B3 is a double A haplotype, A2A1, where the A1 is unusual in possessing strong haemolytic activity, or, alternatively, it is truly an A2B3 haplotype where the B3 is an unusual Ch- product of the B-locus.

Until recently it was necessary to rely on the somewhat unsatisfactory criterion of haemolytic activity after neuraminidase treatment to distinguish A and B gene products with intermediate electrophoretic positions. Roos et al. (1982a) have presented a new electrophoretic technique for separating C4 variants. Reduced immunoprecipitates are separated by SDS polyacrylamide gel electrophoresis into component α-, β-, and γ-chains. Phenotypically A3BQO plasmas have α-chains of slightly higher molecular weight than α-chains of AQOB1 plasmas, and

plasmas with A and B products show both chains. Furthermore, the α-chain of the most common A-gene product, A3, reacts with anti-Rg serum and the α-chain of B1 reacts with anti-Ch serum. Roos (1982b) has further reported an unusual C4 gene-product which was classified as a B allotype by IEP, functional activity, and family segregation, but was Ch-. In the SDS technique, this variant had the B-type α-chain, indicating that Ch- B-products exist. This technique will be useful for assigning seemingly identical, Ch or Rg different, variants to A or B loci.

d. The problem of uninformative A3B1 phenotypes

Many individuals are phenotypically A3B1 and cannot be genotyped since it is impossible to exclude a null allele at one or two of the four C4 loci. A number of investigators (notably Awdeh et al., 1979) have used quantitative or semi-quantitative measures of the relative amounts of A and B protein per phenotype to assign genotypes to A3B1 individuals. It can be seen from Figure IV-10 (p. 105) that A3B1 plasmas do not always clearly display the 1:2, 1:1, and 2:1 ratios of C4 A:B protein expected if each phenotype is a combination of either one A and two B genes, one A and one B gene, two A and two B genes, or two A and one B genes.

An attempt was made to determine the sort of distribution which might be expected from a sample of 154 A3B1 phenotypes if the sample were a mixture of 0.5, 1.0, and

2.0 curve ratios. In order to create a model distribution it was necessary to (i) estimate the number of individuals in the sample expected to fall into each of the three groups, and (ii) estimate the standard deviation of each ratio group.

To estimate the number of each genotype expected, the haplotype frequencies published by Raum et al., (1980, Table V-1, p. 207) were applied to the sample of 154 individuals tested using the assumption, as these were selected A3B1 phenotypes, that only the genotypes A3B1/A3B1, AQOB1/A3BQO, A3BQO/A3B1, and AQOB1/A3B1 were occurring. The expected numbers obtained are 25 AQOB1/A3B1 genotypes (A:B curve ratios = 0.5), 111 A3B1/A3B1 + A3BQO/AQOB1 (A:B curve ratios = 1.0) and 18 A3BQO/A3B1 (A:B curve ratios = 2.0). These are a very crude estimates and likely to overestimate three-gene and two-gene phenotypes, as the homozygotes AQOB1/AQOB1 and A3BQO/A3BQO were not observed in the families from which the individuals were selected.

To estimate the standard deviation, it was assumed that the sample contained three groups, each symmetrically distributed around mean ratios of 0.5, 1.0, and 2.0. In the first group with a mean ratio of 0.5, the lower limit is 0 and the range therefore likely to be 0 - 1. The standard deviation, taken at 1/6 of the range, is 0.17. Adjustment of this variance for the 1.0 and 2.0 groups is made

according to calculations elaborated by Young (1962), giving 0.17 x 1.26 = 0.21, and 0.17 x 2.0 = 0.34 as the estimates of the standard deviations of the 1.0-ratio and 2.0-ratio groups respectively.

Figure V-1 compares the model distribution obtained using these assumptions, with the distribution actually observed, after the two outliers (4.32 and 5.78) are removed. Chi square for observed/expected is 16.86, p = .30. This indicates a reasonably good fit between the distribution of A:B ratios obtained and the model proposed. It can be seen from Figure V-1, however, that fewer 1.0 individuals and more 0.5 and 2.0 individuals in the expected distribution would increase the similarity between the two. Also, it is an underlying assumption here that each C4*A3 gene produces an amount of C4 equal to that produced by each C4*B1 gene. Preliminary studies on half-null homozygotes, A3BQO/A3BQO and AQOB1/AQOB1 individuals, for example, suggest that this is probably not so.

The model described is only one of a number of possible ones and proves nothing definite about the sample of A3B1 individuals tested here but two tentative conclusions can be drawn. First, the curve ratios obtained do show a distribution consistent with the possibility that there are three groups of individuals in the sample with curve ratios of 0.5, 1.0, and 2.0. The distribution

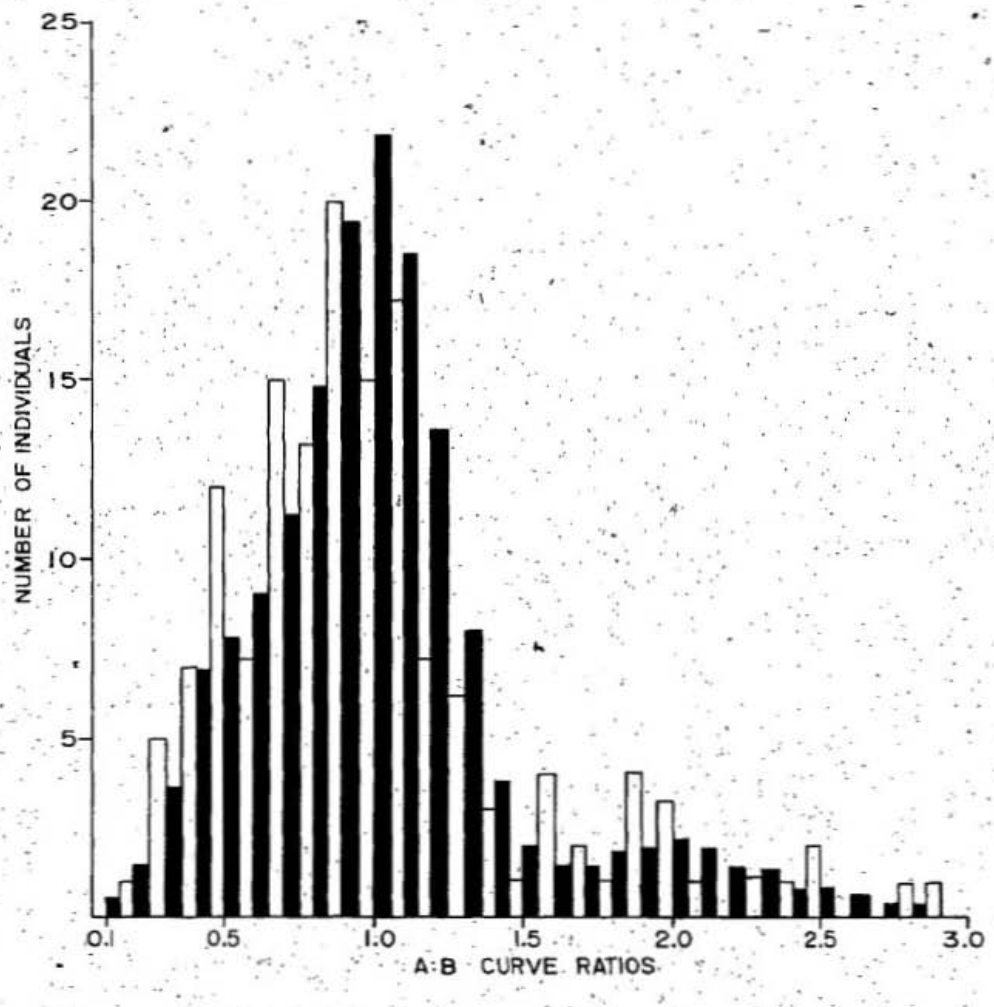


Figure V-1: Observed (□) and expected (■) distributions of C4 A:B curve ratios after crossed immunoelectrophoresis of 152 A3B1 plasmas. Two outliers have been removed from the observed distribution shown in Figure IV-10.

obtained does not, in other words, exclude a possible gene dose effect within phenotypes. Second, and more important, there is considerable overlap between 0.5 and 1.0 groups in both the model and observed distributions. Therefore, while there may be three statistically apparent groups in a population sample, as Awdeh et al. (1979) have suggested, the utility of the CIE method for assigning genotypes to individuals is definitely limited.

e. Haplotype and gene frequencies

There were 1048 founder haplotypes of which 603 were completely typed. An additional 445 were incomplete or partially haplotyped, that is, had one locus or both for which two interpretations, including the existence of a null allele, were possible.

Of the 25 two-locus combinations possible from five C4*A and five C4*B alleles, only 14 were observed. Two additional haplotypes, A3A2BQO and A2B2B1, were postulated to contain products of three loci. Six completely-typed AB pairs of alleles show linkage disequilibria and five of the of these pairs account for 90% of the total complete haplotypes observed. In Table V-1, the frequencies obtained for each haplotype are compared with frequencies published by Raum et al. (1980). The proportions of A3B1, A3BQ0, and AQOB1 in the two sets of data differ markedly. This is to be expected since in this study approximately 46% of these three haplotypes have been classified A3*B1*, A3*B1, and

Table V-1: Comparison between C4 haplotype frequencies obtained by direct count from 1046 founder haplotypes and C4 haplotype frequencies published by Raum et al. (1980)

C4 haplotype	Frequency	
	This study	Raum et al. (1980)
a. A3B1	.225	.546
AQOB1	.113	.123
A3BQO	.085	.088
b. A3*B1*	.164	
A3B1*	.098	
A3*B1	.094	
Total a+b	.779	.757
c. A4B2	.070	.065
A6B1	.024	.050
A4B4	.014	
AQOB2	.009	.038
A2B2	.009	.019
A2B1	.008	.035
A3B3	.008	.004
A2BQO	.006	.004
A3B2	.002	.015
AQOB3	.001	
A2B3	.001	.004
A1BQO		.004
A1B1		.004
d. A3A2BQO	.003	
e. A2B1*	.012	
A2*B1	.002	
A2B2*	.001	
A4*B2	.001	
A3*B2	.010	
A3*B3	.011	
A3B3*	.001	
f. A3*A2BQO	.010	
A3*A2B1*	.007	
A2*A3BQO	.005	
A3*A2B2*	.001	
A2B2B1*	.007	
A2B1B2*	.003	
A3*A2B1*	.001	
Total c+d+e+f	.227	.242

A3B1* by me (category b in Table V-1). The proportion of haplotypes containing A3 or AQ0 and B1 or BQ0 (a+b in Table V-1) is similar in the two studies; 78% in this study and 76% in the data reported by Raum and coworkers. If the two populations from which these data are derived have similar frequencies of A3B1, AQ0B1, and A3BQ0 haplotypes, then 90-100% of the incomplete haplotypes (b in Table V-1) must be A3B1. This is surprising in view of the results given in Figures IV-10, p. 105 and V-1, p. 205. When 154 samples phenotyped A3B1 were subjected to CIE, only 64% (A:B curve ratio = 1 ± 3 S.D.) showed approximately equal concentrations of A3 and B1 antigens.

An alternative interpretation is that these populations differ. More than half the individuals bled for this investigation came from genetically isolated communities in Newfoundland and Labrador and are mainly of English and Irish descent. Approximately 60% of the sample were patients and their families. Raum's data came from healthy, Caucasoid, presumably urban, North Americans in the Boston area who are likely to be a more heterogeneous population. Only the haplotype A4B2 has similar frequencies in the two groups. A6B1 appears to be decreased in the individuals used for this study, particularly in the WP (Winnipeg) and MS (London, Ont.) groups. Haplotypes containing the variant A1 were not observed. Neither A4B4 nor A3B3 was reported by Raum. For haplotypes containing A2 and/or B2 the two studies cannot be compared, since approximately 80% of the

rare incomplete haplotypes (e and f in Table V-1) described by me contain one or both of these gene products.

Table V-2 shows allele frequencies obtained (i) by direct count from the founder haplotypes in this study, (ii) by estimating A3, AQO, B1, and BQO alleles from complete and incomplete haplotypes (see Chapter IV, p. 125) and (iii) by extrapolation from Raum et al. (1980 and Table V-1). The figures given in column (i) are likely to be accurate for the alleles A6, A4, A2, B4, B3, and B2 since typing was definite for all but at most 1% of these. A2 and B2 have similar frequencies at 6 and 11-14% respectively in both studies. A6 occurs less frequently in the populations examined in this study (2.3% cf 5%), whereas A4, B3, and B4 are more frequent.

Comparing estimates (column ii) of the common alleles with the published data indicates that A3, AQO, and B1 have relatively similar frequencies. BQO is more frequent in this study with an uncorrected frequency of 11% (column i) and an estimated frequency of 16% (column ii) as compared with 10% in the Raum data. This difference is most evident in the WC sample where 17% of the founder haplotypes of this large kindred carry the allele BQO. Furthermore, 17% is a conservative estimate since a large proportion of this population (40%) was typed B1* and some of these must be BQO.

These comparisons must be treated cautiously, however,

Table V-2: Frequencies for C4*A and C4*B alleles obtained (i) by direct count from total haplotypes observed in this study(1), (ii) by estimates from total complete and incomplete haplotypes (2), and (iii) by extrapolation from haplotype frequencies published by Raum et al. (1980) (3).

C4 allele	Frequency (i) this study	(ii) this study	(iii) Raum et al.
A3	.410	.640	.653
AQO	.120	.180	.161
A6	.023		.050
A4	.082		.065
A2	.063		.062
A1			.008
A4*	.001		
A3*	.289		
A2*	.007		
B1	.463	.690	.758
BQO	.107	.160	.096
B4	.014		
B3	.020		.008
B2	.105		.137
B1*	.286		
B2*	.006		
B3*	.001		

(1) Includes alleles from 26 founder haplotypes which are likely to have 2 C4*A alleles/haplotype and 11 which are likely to have 2 C4*B alleles.
(2) See Chapter IV, pp. 125, 128.
(3) See Table V-1, p. 207.

since, as was stated earlier, a large proportion of the individuals which comprised the families studied here were selected because they contained individuals with disorders such as multiple sclerosis, myotonic dystrophy, Hodgkin's disease, immunodeficiency disorders, or individuals who were prospective kidney or bone marrow recipients. Furthermore, comparing frequencies obtained by different investigators may be premature at this time for a variety of reasons. The naming system, particularly for rare variants is not uniform. There is no method for distinguishing A3BQO/AQOB1 from A3B1/A3B1 genotypes, and the published method of distinguishing among A3B1/A3BQO, A3B1/A3B1, and A3B1/AQOB1 genotypes is not satisfactory.

B. Association data

In this study, associations among MHC alleles, with particular emphasis on C4 associations, were calculated in order to confirm already published associations for these populations, to uncover previously unreported associations, and to determine whether the values obtained could be used to speculate on the relative position on the chromosome of the C4 and other complement genes or on the evolution of the alleles.

1. Frequencies of MHC alleles

Frequencies of BF and C2 alleles in the populations

studied are similar to those reported elsewhere (see Chapter I). The frequencies of HLA-B antigens are similar to those published for North American and European Caucasoids (see Table IV-17). The exceptions are HLA-B8 which occurs in approximately 13% of the haplotypes in this study, B12 which occurs in 23% of the WC (West Coast, Nfld.) population, and B7 which occurs in 17% of the total haplotypes and in 22% of the MS (multiple sclerosis, London, Ont.) series. In addition, HLA-DR2 and DR3 appear to be increased in the MS family haplotypes (Table IV-18, p. 141).

The point has been made earlier that a sizable number of the individuals sampled for this investigation were selected because they or their relatives were patients, and another sizable number were from the island of Newfoundland, many from genetically-isolated communities. Differences in frequencies are therefore not surprising and are, in fact, to be expected.

2. Pair-wise associations between MHC alleles other than C4.

These were ascertained by both chi square and delta standard methods and, of the two, the chi square method was more restrictive. Thirty-three pairs of alleles had standard delta values of greater than 25%. Thirty-two pairs had probability values of less then 5% (Fisher's exact test), but only 15 pairs were significant after probability

values were corrected for the number of comparisons made. In general, linkage disequilibria for alleles other than C4 (Tables IV-20 and IV-21, pp. 145, 146) confirmed those already published.

3. Pair-wise associations between C4 haplotypes and alleles of the other MHC loci

These calculations were complicated by the fact that a null allele could not be excluded from 445 of the total 1048 haplotypes included in the study. Chi square and delta standard values were therefore calculated twice, first using complete C4 haplotypes only, and then using the total of all complete and incomplete haplotypes. Interpreting the results is therefore difficult because values for the same pairs vary somewhat in the two sets of calculations. To simplify interpretation, it is useful to consider four categories of haplotypes:

a. For haplotypes in which a null allele could be excluded in all, or nearly all, cases, values based on the larger total (e.g. N=1036 for HLA-B/C4) are likely to be more exact. The haplotype A4B4 is such a case since no haplotypes were classified A4*B4 or A4B4*. There are seven significant pairwise associations in this category, namely HLA-B14/A2B2, HLA-B18/A4B2, BF*S/A4B2, C2*2/A4B2, HLA-Bw22/A4B4, C2*2/A4B4, HLA-B17/A6B1, and HLA-DR7/A6B1. At least three of these have been reported by others.

Only for HLA-B18/A4B2 is there a fairly large

discrepancy between values obtained in the two sets of calculations. Eleven B18 haplotypes classified A3*B1* were included in the larger total but obviously absent from the smaller one which included complete haplotypes only.

b. A second category includes haplotypes other than AQOB1, A3BQO, and A3B1, in which a null allele could not be definitely assigned or excluded in all cases. The haplotype A2B1 BF*S1 is one such example. This combination occurred four times. The larger total included an additional five haplotypes which were A2B1* BF*S1, and values for the A2B1–S1 pair are lower in the second set of calculations. If all A2B1* S1 haplotypes are assumed to be A2B1, then the standard delta value becomes 81% (χ^2= 312.66, p<.4 x 10^{-14}). Significantly associated pairs in this category, besides A2B1/S1, are A3B3/HLA-B15 and A2B2/DR1.

c. AQOB1, A3BQO, and A3B1 haplotypes are a special problem since the vast majority of incomplete haplotypes are of these three types and their frequencies are distorted in both totals. Relative delta values for the B8–AQOB1 pair, for example, are nevertheless similar for both sets of calculations, being 81% when N=592 and 75% when N=1036. Thirty-three of 1036 haplotypes were B8 A3*B1* + A3*B1. If all these are assumed to be AQOB1, then the total B8–AQOB1 haplotypes becomes 125 and relative delta = 82%. This is a likely overestimate of the total AQOB1, however, but the strength of association is not affected greatly. Other

pairs in this category showing linkage disequilibria are AQOB1/BFS, AQOB1/C2*1, AQOB1/DR3, A3BQO/B12, A3BQO/BF*F1, A3B1/B7, A3B1/C2*1, and A3B1/DR2.

d. Putative double A and B haplotypes have all been scored with an asterisk, except for three definite A3A2BQO. Significantly associated pairs which include double A haplotypes are A3*A2BQO/BF*F, A3*A2BQO/HLA-B35, A3*A2BQO/B12, and A3*A2B1*/B35. Of the three definite A3A2BQO haplotypes, one was B12, C2*1, BF*F, DR NT and two were B35 C2*1 BF*F DR NT. If all incomplete double A haplotypes are assumed to be A3A2BQO then the relative delta for A3A2BQO/B35 is 49%, for A3A2BQO/BF*F is 85%, for A3A2BQO/C2*1 is 100%, for A3A2BQO/DR1 is 100%, and for A3A2BQO/B12 is 4%. Of these neither the B12 association nor the association with DR1 is significant. However, the amount of DR data was limited.

The haplotype A2B2B1*, which is likely to contain two B alleles occurred seven times. An additional three haplotypes were scored A2B1B2*. All were B14. Assuming all haplotypes are identical, the relative delta is 100% and the association between this C4 haplotype and B14 is highly significant. Furthermore all ten haplotypes were BF*S and C2*1.

Two points should be emphasized. First, although exact frequencies of some C4 alleles are impossible to determine using present typing methods, many of the pairwise

associations that occur are highly significant and are affected very little by the distortions caused by the problem of hidden null alleles. Second, the correlation between values obtained by delta standard and chi square methods is, certainly for the C4/MHC associations, fairly high. Thirty-six pairs gave relative deltas of 25% or higher. Of these, 23 pairs were highly significant by Fisher's exact test, and a further 11 pairs, while not significant after correction for number of comparisons, had p values $< .01$.

Furthermore, the significance levels used here may be overly restrictive. Table V-3 summarizes appropriate levels of significance as suggested by Piazza (1975) and reiterated by Grange et al. (1981) necessary to pick out non-random pairwise associations. Using these criteria, 23/32 HLA-B, -DR, BF, and C2 pairs and 31/36 C4-MHC pairs selected by relative deltas are significantly associated.

4. Complotypes

Grange et al. (1981) have compared the delta standard method as a means of selecting multiple component associations, with the method of factorial correspondance analysis. They found a fairly good statistical correlation between the two methods. For this reason, and because calculation of chi squares and probabilities is very cumbersome for multiple associations, relative deltas only were calculated for three-, four- and five-way associations. Combinations

Table V-3: Significance levels necessary to select nonrandom 2 x 2 associations (1).

HLA loci	Number of possible associations	Number of associations randomly significant with a probability of 5%	Significance level necessary to pick out nonrandom associations
B-C4	19 x 32 = 608(2)	30	< 0.0017
B-C2	19 x 2 = 38	2	< 0.025
B-BF	19 x 4 = 76	4	< 0.0125
B-DR	19 x 9 = 171	9	< 0.0056
C4-C2	32 x 2 = 64(2)	3	< 0.0166
C4-BF	32 x 4 = 128(2)	6	< 0.0083
C4-DR	32 x 9 = 288(2)	15	< 0.0033
BF-C2	4 x 2 = 8	1	< 0.05
BF-DR	4 x 9 = 36	2	< 0.025
C2-DR	2 x 9 = 18	1	< 0.05

(1) Adapted from Grange et al., 1981.
(2) Using all complete and partial C4 haplotypes. For 14 complete C4 haplotypes only, $p < 0.004$ for B-C4, $p < 0.025$ for C4-C2, $p < 0.02$ for C4-BF, and $p < 0.008$ for C4-DR.

with relative delta values of $\geq$ 25% have been taken as likely to be occurring more often than expected from the frequencies of their component alleles. Although these combinations will be referred to occasionally in this discussion as likely linkage disequilibrium combinations, it must be emphasized that Ds values are not linkage disequilibrium parameters in the strict sense. Delta values refer only to the difference between observed and expected haplotype frequencies and do not take into account two-, three-, or four- (etc.) loci linkage disequilibria among the alleles within a haplotype.

Awdeh et al. (1983) and Dawkins et al. (1983) have suggested the term "complotype" to designate combinations of C4, BF, and C2 alleles inherited as gametic units. In this study, relative delta values of 25% or greater have been taken as identifying three-way combinations of complement alleles, or complotypes, likely to be occurring more frequently than expected from their individual frequencies. At least 14 possible linkage disequilibrium combinations were selected in this way. Calculations were based on the total of all complete and incomplete haplotypes for which C4, BF, and C2 typing data were available (N=861).

As with the pairwise associations, results were complicated by the presence of incomplete C4 haplotypes. Only A3B1-S-1 and A3BQO-F-1 had relative deltas of less

than 50%, at 33% and 28% respectively. These values may be slightly depressed by the presence of A3*B1*, A3B1*, and A3*B1 haplotypes in the total. For example, a large proportion of A3*B1 and A3*B1* must be A3B1, so the frequency of A3B1-S-1 is higher than it appears here. Furthermore, 46% of A3B1* haplotypes are Bf*F, C2*1, as compared to 20% of known A3B1. This suggests that a proportion of A3B1* are in fact A3BQO-F-1.

Awdeh et al. (1983) have identified 14 complotypes occurring in their material with frequencies of .01 or greater. Table V-4 compares these complotypes to the combinations observed in this study. For the complotypes observed by me, frequencies are also shown, as are relative deltas where those values are greater than 25% (from Table IV-28).

Of the 14 complotypes observed by Awdeh et al., all but one, AQOB2-F-1, also occur in this material with frequencies of greater than .01 if one assumes that the complotypes bracketed in the figure are identical. Eight of the complotypes identified by Awdeh et al. show likely linkage disequilibria, as measured by relative delta, in this material. Six do not. Five additional combinations, A4B4-S-1, A2B1/BQO-S1-1, A2BQO-F-1, and the putative "double C4" complotypes A2B2B1-S-1 and A3A2BQO-F-1 occur in this material more frequently than might be expected from the component allele frequencies. The existence of such a

Table V-4 : Complement allele combinations observed in this study and 14 complotypes with frequencies > .01 reported by Awdeh et al. (1983).

This study					Awdeh et al. (1983)			
BF	C2	C4	Freq.	Ds	BF	C2	C4A	C4B
S	1	A3B1	.20	32.7				
S	1	A3*B1	.08	54.2	S	C	3	1
S	1	A3*B1*	.13	44.2	S	C	0	1
S	1	AQOB1	.11	88.7				
F	1	A3B1	.04	.....	F	C	3	1
S	1	A3BQO	.05	.....	S	C	3	0
S	1	A4B2	.05	.....	S	C	4	2
F	1	A3BQO	.03	27.7	F	C	3	0
S	1	AQOB2	.01	100.0	S	C	0	2
S	1	A2B1	.005	.....	S	C	2	1
S	1	A2B1*	.003					
F	1	AQOB1	.003	.....	F	C	0	1
S	1	A2B2	.01	56.6	S	C	2	2
S	2	A4B2	.03	59.4	S	B	4	2
S	1	A3B3	.01	100.0	S	C	3	3
S	1	A3*B3	.01	51.0				
S	1	A6B1	.02	81.3	S	C	6	1
S	1	A3B2	.002	.....	S	C	3	2
S	1	A3*B2	.007					
S	1	A4B4	.01	92.1				
S1	1	A2B1	.005	49.0				
S1	1	A2*B1	.005	49.0				
F	1	A2BQO	.003	71.3				
S	1	A2B2B1*	.008	100.0				
S	1	A2B1B2*	.003	100.0				
F	1	A3A2BQO	.003	100.0				
F	1	A3*A3BQO	.003	71.3				
F	1	A3*A2BQO	.009	100.0				

large number of linkage disequilibrium combinations can be taken as further evidence for the very close linkage of the loci for the complement factors C4, BF, and C2.

5. Supratypes

Dawkins et al. (1983) use the term "supratype" to designate extended haplotypes, or combinations of MHC alleles inherited together, as deduced from phenotypes and family studies. Calculations of three-, four-, and five-point associations have been used here to select nonrandomly-associated supratypes. Although the amount of HLA-DR data was small relative to the other loci tested, there were nine five-point associations identified. There were four four-point associations involving HLA-B and the complement alleles only.

Awdeh et al. (1983) have described the distribution of complotypes in relation to HLA-B and HLA-DR. These investigators report eight supratypes or extended haplotypes which show linkage disequilibria. These are HLA-B7 A3B1-S-1 DR2, B8 AQOB1-S-1 DR3, B15 A3B3-S-1 DR4, B17 A6B1-S-1 DR7, B40 AQOB2-S-1 DR6, B14 A2B2-S-1 DR1, B12 A3BQO-F-1 DR4, and B12 A3B1-F-1 DR7.

The first four of these supratypes have been confirmed in this study. Two four-way combinations, B40 AQOB2-S-1 and B14 A2B2-S-1, also show high relative delta values in this material but DR data for these are

incomplete and inconclusive. Three out of six B40 AQOB2-S-1 combinations were DR-typed but, in contrast with the Awdeh supratype, none was DR6. Three out of five B14 A2B2-S-1 combinations were DR-typed and two were DR1. Neither of the two B12 supratypes identified by Awdeh et al., i.e. B12 A3BQO-F-1 DR4 and B12 A3B1-F-1 DR7, showed linkage disequilibria (i.e. Ds $\geq$ 25%) in the material analysed here. B12 was significantly associated with A3BQO, however, and the complotype A3BQO-F-1 occurred 30 times, 14 times on B12. Only two of these 14 combinations were DR-typed, and neither was DR4. Finally, B12 A3B1-F-1 occurred eleven times. Five were DR-typed, and all were DR7.

Six additional supratypes were observed to occur in this material with frequencies greater than .01 and relative deltas greater than 25%. These are B5 A3B1-S-1-DR4, B15 A4B2-S-2 DR4, B18 A4B2-S-1 DR2, B22 A4B4-S-2 DR-, and B35 A3*A2BQO-F-1 DR1. Three positively associated four-way combinations were also identified, namely B18 A3BQO-F1-1, B27 A4B2-S-1, and B14 A2B2B1*-S-1.

6. Position of the C4 loci relative to HLA-B and HLA-DR

Information regarding the position of the C4 loci can be obtained from analyses of recombinant haplotypes and of extended haplotypes or supratypes which show linkage disequilibria.

a. Recombinants

Only four definite recombinant haplotypes were observed in this material. Three were between HLA-A and HLA-B where C4 followed HLA-B in all cases. One was between HLA-B and HLA-DR, where C4 (and C2) followed HLA-DR. Taken alone, these data suggest only that the complement loci are close to HLA-DR. At least twenty B-DR recombinant haplotypes informative for complement genes have been published however (see Chapter I Section C3), and of these the complement alleles follow HLA-B in approximately 50% of the cases and HLA-DR in the other 50%. The C4 loci are likely therefore, to be between HLA-B and HLA-DR, but precisely where, that is, whether closer to B or to DR, is still an open question.

b. Supratype analysis

Some information can be gained from comparing similar supratypes, using the assumption that the most commonly occurring, strongly associated combinations of alleles are ancestral and others, containing some, but not all, of the same alleles have arisen by crossovers between the ancestral forms and other unrelated supratypes. This approach was taken by Olaisen et al (1981). These investigators compared the linkage disequilibrium combination B8-C4*S-DR3 with other haplotypes which differed by one or two alleles. They reasoned that the C4 loci are likely to be between HLA-B and HLA-DR because this position required the fewest

recombinant events to explain variant haplotypes.

Another approach, which follows from this, is to compare relative deltas between pairs of alleles within a supratype, on the assumption that the stronger the association between pairs of alleles, the closer they are likely to be. Ness et al. (1982) have applied this reasoning to the alleles of the supratype HLA-B50 BfS1 DR 3 or 7 and have suggested, based on the magnitude of the relative deltas between B50 and S1, and S1 and DR 3 and 7, that the Factor B locus is closer to HLA-DR.

Table V-5 shows eight supratypes extracted from Table IV-28, that seem to be occurring more frequently than expected (Ds for five-point association > 25%). Relative pairwise delta values for B/C4 and C4/DR associations are also given (from Table IV-24). It can be seen that in five cases out of eight, the C4/DR associations have higher delta values, which suggests, although not conclusively, that the C4 loci are closer to HLA-DR. There are three exceptions. The supratypes HLA-Bw22 C2*2 BF*S C4*A4B4 HLA-DR- and HLA-B18 C2*1 BF*S C4*A4B2 HLA-DR2 place C4 closer to HLA-B, and HLA-B8 C2*1 BF*S C4*AQOB1 HLA-DR3 places C4 equidistant to HLA-B and HLA-DR.

The Bw22 supratype is rare and, furthermore, only five of the twelve Bw22 C2*2 BF*S C4*A4B4 combinations were tested for HLA-DR.

Table V-5: Comparison of relative deltas within eight five-component supratypes.

Supratype B	C2	BF	C4	DR	Allele pairs	%Ds	No. considered
7	1	S	A3B1	2		43.3	
					B7 / A3B1	22.5	71/1036
					A3B1 / DR2	39.6	46/368
8	1	S	AQOB1	3		75.6	
					B8 / AQOB1	75.3	92/1036
					AQOB1 / DR3	74.4	41/368
15	1	S	A3B3	4		100.0	
					B15 / A3B3	73.6	6/1036
					A3B3 / DR4	100.0	3/368
15	2	S	A4B2	4		28.7	
					B15 / A4B2	11.8	12/1036
					A4B2 / DR4	31.1	14/368
17	1	S	A6B1	7		88.7	
					B17 / A6B1	71.1	18/1036
					A6B1 / DR7	89.3	10/368
18	1	S	A4B2	2		35.6	
					B18 / A4B2	19.0	12/1036
					A4B2 / DR2	8.2	9/368
22	2	S	A4B4	-		50.0	-
					B22 / A4B4	79.5	12/1036
					A4B4 / DR-	37.5	3/368
35	1	F	A3*A2BQO	1		57.2	
					B35 / A3*A2BQO	56.0	6/1036
					A3*A2BQO / DR1	68.3	5/368

The B8 supratype will be discussed in a later section.

The combination, B18 C2*1 BF*S C4*A4B2 HLA-DR2, and other similar supratypes are worthy of further consideration. Table V-6 shows all A4B2 haplotypes which were typed for HLA-B and HLA-DR, arranged according to HLA-B. Table V-7 shows the same haplotypes arranged according to HLA-DR. The various combinations appear to be less variable with respect to HLA-DR than to HLA-B. In fact, 90% of the supratypes shown here are three of ten possible DR types, 2,4, and -, whereas 90% are eight of nineteen possible HLA-B types. This is to be expected, however, if the combined allele frequencies of the three DR alleles and of the eight B alleles are similar in the population as a whole. In fact, DR2 + DR4 + DR- occurs with a frequency of 47% in world data (Bauer and Danilovs, 1980) and 46% in this study. The combined frequencies of the eight B alleles (15, 35, 22, 5, 40, 18, 27, 7) is 49% in world data and 56% in this study. There is clearly not much to choose between here, suggesting that B-C4 and C4-DR are recombining at approximately equal rates.

Another approach is to compare the strength of the association between A4B2 and the eight B alleles with that between C4 and the three DR alleles. The 2 x 2 tables in Table V-8 show the distribution of A4B2 in the population studied, taking the three DR alleles and the eight B alleles as a unit in each case. What is interesting are the

Table V-6 : Five-component supratypes containing the C4 haplotype A4B2 arranged according to HLA-B.

Complotype A4B2-S-2					Complotype A4B2-S-1				
B	C2	BF	C4	DR	B	C2	BF	C4	DR
15	2	S	A4B2	4					
15	2	S	A4B2	4					
15	2	S	A4B2	4					
15	2	S	A4B2	4					
15	2	S	A4B2	4					
15	2	S	A4B2	2	15	1	S	A4B2	2
15	2	S	A4B2	2	15	1	S	A4B2	2
-	2	S	A4B2	4					
35	2	S	A4B2	4	35	1	S	A4B2	1
35	2	S	A4B2	4					
22	2	S	A4B2	4					
22	2	S	A4B2	2					
5	2	S	A4B2	4	5	1	S	A4B2	4
40	2	S	A4B2	-	40	1	S	A4B2	4
					18	1	S	A4B2	2
					18	1	S	A4B2	2
					18	1	S	A4B2	2
					18	1	S	A4B2	2
					(18	0	S	A4B2	2)
					27	1	S	A4B2	2
					27	1	S	A4B2	4
					27	1	S	A4B2	4
					27	1	S	A4B2	-
					27	1	S	A4B2	-
					37	1	S	A4B2	5
					16	1	S	A4B2	4
					7	1	S	A4B2	4
					7	1	S	A4B2	1
					7	1	S	A4B2	-

Table V-7 : Five-component supratypes containing the C4 haplotype A4B2, arranged according to HLA-DR.

Complotype A4B2-S-2					Complotype A4B2-S-1				
DR	C4	BF	C2	B	DR	C4	BF	C2	B
4	A4B2	S	2	15	4	A4B2	S	1	27
4	A4B2	S	2	15	4	A4B2	S	1	27
4	A4B2	S	2	15	4	A4B2	S	1	5
4	A4B2	S	2	15	4	A4B2	S	1	40
4	A4B2	S	2	15	4	A4B2	S	1	16
4	A4B2	S	2	-	4	A4B2	S	1	7
4	A4B2	S	2	35					
4	A4B2	S	2	35					
4	A4B2	S	2	5					
4	A4B2	S	2	22					
2	A4B2	S	2	15	2	A4B2	S	1	15
2	A4B2	S	2	15	2	A4B2	S	1	15
2	A4B2	S	2	22	2	A4B2	S	1	18
					2	A4B2	S	1	18
					2	A4B2	S	1	18
					2	A4B2	S	1	18
					(2	A4B2	S	0	18)
					2	A4B2	S	1	27
-	A4B2	S	2	40	-	A4B2	S	1	27
					-	A4B2	S	1	27
					-	A4B2	S	1	7
					1	A4B2	S	1	7
					1	A4B2	S	1	35
					5	A4B2	S	1	37

Table V-8: Contingency tables and relative delta values for the associations between (a) C4*A4B2 and eight HLA-B alleles combined and (b) C4*A4B2 and three HLA-DR alleles combined.

(a)

		C4*A4B2 +	C4*A4B2 −	
HLA-B15 + B35 + B22 + B5 + B40 +B18 + B27 + B7	+	58	520	χ^2 = 19.62 p = .15 x 10^{-5} Ds = 58.7%
	−	13	445	

(b)

		C4*A4B2 +	C4*A4B2 −	
HLA-DR2 + DR4 + DR3	+	28	151	χ^2 = 19.52 p = .18 x 10^{-5} Ds = 75.6%
	−	4	185	

relative delta values obtained in this way. Ds for C4*A4B2 and HLA-DR 2 + 4 + / - is 75.6%, whereas Ds for C4*A4B2 and the eight B alleles combined is 58.7%. If relative strength of association can be taken as evidence of position, then the C4 loci appear to be closer to HLA-DR.

7. The order of complement loci

The A4B2 supratypes in Tables V-6 and V-7 have been separated according to C2 alleles, those on the left containing C2*2 and those on the right containing C2*1. The supratypes containing C2*1 are clearly more variable with respect to HLA-B and HLA-DR alleles than are the C2*2 supratypes. If such variability can be taken as a measure of relative age, then the C2*1 variant is likely to be the ancestral form from which the C2*2 allele arose, perhaps by point mutation. This is further supported by the fact that the C2*1 allele is common in Caucasoid populations while the C2*2 is relatively rare.

One possibility is that the mutation from C2*1 to C2*2 arose on a B15 C2*1 BF*S C4*A4B2 DR2 or DR4 supratype and became distributed to other supratypes by crossing over between HLA-B and C2 and between C2 and HLA-DR. This implies, because of the close association between C4*A4B2 and DR 2, 4, and - in both C2*1 and C2*2 supratypes, that the C4 loci (and possibly also BF) are closer than the C2 locus to HLA-DR.

8. Evolution of MHC supratypes

Table V-9 shows all the supratypes observed in this material which contain the two rare C4 haplotypes, A4B4 and A3B3. Only haplotypes for which typing is definite for both A and B loci are given. There are a number of interesting similarities between these supratypes and those which contain A4B2 (Tables V-6 and V-7). Of the total (57), 15 contain B15, and this allele is found with A4B2-S-2, A4B2-S-1, and A3B3-S-1. Fifteen contain Bw22, and this allele is found with A4B2-S-2, A4B4-S-2, and A3B3-S-1. DR4 appears on 22 of 40 supratypes tested, with A4B2-S-2, A4B2-S-1, A4B4-S-2, and A3B3-S-1. Seven supratypes are DR- and these also include A4B2-S-2, A4B2-S-1, and A4B4-S-2. Furthermore, 22 of 55 complete supratypes (12 varieties) contain two unusual complement variants on the same supratype, namely A4B2 and C2*2, A4B2 and C2*0, A4B4 and C2*2.

If one assumes that (a) shared alleles imply relatedness, (b) the complement loci are situated between HLA-B and HLA-DR, and (c) differences have arisen by mutation, crossing over, and recombination, no easily recognizable pattern of descent emerges. C2*2, for example, is likely to have originated by mutation of C2*1, and the most probable candidate for an ancestral haplotype is B15 C2*1 BF*S C4*A4B2 DR4. Yet this haplotype has not been observed in this material. Similarly B4 may have arisen as a mutation

Table V-9 : Supratypes containing A4B4 and A3B3.

A4B4					A3B3 (1)				
HLA-B	C2	BF	C4	DR	HLA-B	C2	BF	C4	DR
7	1	S	A4B4	NT (2)	15	NT	NT	A3B3	NT
7	2	S	A4B4	NT	15	1	S	A3B3	NT
22	NT	S	A4B4	NT	15	1	S	A3B3	4
22	2	S	A4B4	NT	15	1	S	A3B3	4
22	2	S	A4B4	NT	15	1	S	A3B3	4
22	2	S	A4B4	NT	15	1	S	A3B3	4
22	2	S	A4B4	NT	22	1	S	A3B3	NT
22	2	S	A4B4	4 (3)	18	1	F	A3B3	NT
22	2	S	A4B4	4					
22	2	S	A4B4	-					
22	2	S	A4B4	-					
22	2	S	A4B4	-					
22	2	S	A4B4	3					
22	2	S	A4B4	5					
40	2	S	A4B4	?					

(1) A further eleven C4 haplotypes have been designated A3*B3 because a null allele could not be excluded at the A locus. Six were B15 C2*1 BF*S DR(NT). Two were B15 and not tested for C2, BF or DR. One was B40 C2*1 BF*S DR(NT) and one was B5 C2*(NT) BF*S, and one was B37 C2*1 BF*F DR(NT).

(2) NT = not tested

(3) This supratype was found in a family selected for B27 as part of an ankylosing spondylitis project and was not included in the total haplotypes used for this study.

on a Bw22 C2*2 BF*S A4B2 DR4 supratype, but this one has also not been observed. Furthermore, no one order of complement genes with respect to HLA-B and HLA-DR gives a simple, consistent pattern of recombinational events which might account for the present assortment of alleles within these supratypes.

The relatively small amount of data may account for this confusion. Almost no supratype frequencies have been published, but the small number of A4B2 and A3B3 supratypes described by Awdeh et al. (1983) appear to be relatively similar in their component alleles. It is tempting to speculate that the classical rules of mutation and crossing over are not the only ones operating however, and that other genetic mechanisms have played a role in the origin and evolution of these combinations.

Pease et al. (1983) and Weiss et al. (1983) have recently reported data on murine H-2 mutants which suggest that a copy mechanism analogous to gene conversion may play an important role in the generation of polymorphism of these genes. C4 genes are located within the HLA-region, the human analog of H-2. C4 genes are highly polymorphic, and the increasing number of reports of new variants suggests a degree of diversity which rivals HLA. A gene conversion mechanism could explain the existence of moderately rare C4 alleles like A4, B4, B3, B2, on relatively similar B-DR supratypes. Gene conversion

provides a possible means by which new DNA sequences can arise within genes or by which mutated sequences can spread from one chromosome to another, but which, unlike classical crossing over, does not disturb the rest of the gene or the rest of the haplotype. Such a mechanism could also account for the apparent Chido and Rodgers variability observed for seemingly identical C4*B3 and C4*B4 alleles.

Table V-10 shows the MHC supratypes containing BF*F1 and BF*S1. F1 occurs on MHC supratypes containing C4BQO and S1 on supratypes containing C4*A2, both being unusual C4 alleles. Similarly, as stated above, C2*2 (Tables V-7 and V-9) occurs with C4*A4B2 and A4B4, and C2QO occurs with A4B2. In each of these examples we are seeing a clustering of two or more rare, or moderately rare complement alleles on the same supratype. This suggests that, either these complement loci are sufficiently close together to be modified by the same mutational event, or that the MHC regions of some chromosomes are hypermutable, or both.

Natural selection may also be important in supratype evolution. It is possible that certain combinations are subjected to positive selection pressures by virtue of their linkage to important immune response genes. Present data only provides evidence for negative selection pressures on many rare, or moderately uncommon, complement genes. Rittner and Bertrams (1981) have suggested that many MHC-associated diseases are characterized by their

Table V-10: Supratypes containing BF*F1 and BF*S1.

BF*F1					BF*S1				
B	BF	C2	C4	DR	B	BF	C2	C4	DR
18	F1	1*	A3BQO	NT	12	S1	1	A2B1	NT
18	F1	NT	A3BQO	NT	12	S1	NT	A2B1*	NT
18	F1	1	A3BQO	NT	12	S1	1*	A2B1	NT
18	F1	NT	A3BQO	NT	12	S1	1*	A2B1*	NT
18	F1	1*	A3BQO	?					
					17	S1	1*	A2B3	-
14	F1	1	A3BQO	3					
					21(50)	S1	1*	A2B1*	7
					21(50)	S1	1*	A2B1*	NT
					21	S1	NT	A2B1*	NT
					27	S1	1*	A2B1*	NT
					27	S1	1*	A2B1*	NT
					42	S1	1*	A2B1	3

associations with rare complement variants. Support for this hypothesis comes from the reported associations between IDDM and rare C4B variants (see Section C, this chapter), between congenital adrenal hyperplasia and rare C4B variants, and the various associations reported between various disease states and HLA-B8 C4*AQOB1.

Although these associations are likely to be secondary to associations with disease susceptibility genes within these supratypes, the possibility does exist that, in some cases, inherited complement variants may be directly important. For example, the association between systemic lupus erythematosus (SLE) and HLA-B8 was reported some years ago. Many SLE patients are known to have inherited complement deficiencies, particularly of C4 and C2 (Lachmann and Hobart, 1978b). An association has recently been demonstrated between SLE and C4 AQO and BQO, which is of greater magnitude then that between HLA-B8 or DR3, implying that SLE patients are more likely to possess partial null C4 genotypes than are healthy individuals (Fielder et al., 1983). C3b-receptors have been shown to be polymorphic (Wong et al., 1983; Dykman et al., 1983) and in some individuals, partially deficient (Wilson et al., 1982). SLE patients are more likely to be partially deficient for these receptors than healthy individuals (Wilson et al., 1982). Finally, C3b receptors have been shown to function directly in the complement pathway, as cofactors for the degradation of cellbound C3b by C3b-Ina

Whether these observations are linked, or how they are linked, to the pattern of disease which is SLE, is unknown. C4 and C2 form the classical C3 convertase which produces C3b. C3b plays a role in the formation of immune complexes; and C3b-receptors are likely to be important in the degradation of C3b. There may be a single underlying mechanism at work here, or C4, C2, or C3b-receptor deficiencies may be separate routes to the same disease symptoms. In either case, the patterns of association described certainly suggest a direct role for inherited complement variants in SLE.

There is presently little evidence for positive selection pressures on particular MHC alleles or supratypes. The frequent appearance of more than one rare complement allele in the same supratype, and of different rare alleles on similar supratypes, raises a number of interesting possibilities. Perhaps a single mutational event has modified more than one of a group of tandemly arranged genes. Perhaps electrophoretically different complement variants are also functionally different and some combinations exhibit greater fitness than others. Perhaps there is, in some haplotypes, a direct functional relationship between one or more complement alleles and other alleles of the MHC, so that certain combinations are favoured while others are not.

One final mechanism will be considered. The B8

supratype has been described by a number of investigators. It is one of the two most commonly occurring combinations in this material, and the associations, particularly between HLA-B8 and C4AQOB1 and between C4AQOB1 and DR3 are striking. Only 21 out of 117 AQOB1 haplotypes in this material are not B8. Only 11 of 52 AQOB1 haplotypes tested are not DR3. Alper et al. (1982) and Awdeh et al. (1983) have suggested that certain linkage disequilibrium supratypes or extended haplotypes are human analogs of the murine t mutants. The T/t region extends its influence through the murine MHC region and is associated with crossover suppression and a positive male transmission bias. To support their hypothesis, these investigators cite the strong association between alleles of a B8 supratype and the marked transmission bias they have found in the same supratype, namely HLA-A1 B8 BF*S C2*C C4*AQOB1 HLA-DR3 GLO2. The strong linkage disequilibrium found in this study and the relatively similar delta values among component alleles may lend further support to this hypothesis, although the B8 supratypes described in this study have not yet been analysed for the products of the GLO locus.

Such a mechanism may have been important in maintaining some of the rare combinations described in this material. Rare variants may arise and become locked into supratypes, or fragments of supratypes, by suppressed recombination. Such a mechanism might explain, for example, the continued existence of such combinations as B22-2-S,

B15-2-S, C2*2-S-DR4, and C2*2-S-A4B2-DR4, etc., in a variety of slightly different supratypes.

C. Disease associations

Approximately 360 haplotypes were collected from multiplex multiple sclerosis families and 28 from nuclear families with one case of insulin-dependent diabetes mellitus in order to investigate the possible relationship between particular C4 and other MHC allotypes and these diseases.

1. Multiple sclerosis

The pedigree data and diagnostic criteria used in these MS families have already been published (Ebers et al., 1982). These investigators have shown that, contrary to expectation, haplotype-sharing, specifically with regard to HLA-A, HLA-B, and HLA-DR alleles, differed little from that expected by chance in 40 MS sib-pairs from 36 families. Thus the hypothesis that a single, MHC-linked disease-susceptibility gene is operating in this disease was not supported by this study.

A different approach has been taken in this analysis. From the pedigrees, 188 entry or founder haplotypes, or supratypes, could be identified as MS supratypes by the fact that they occurred in the patients. A further 162

supratypes were observed in the healthy relatives of the patients, but not in the patients themselves. The latter number is smaller because, although three generations of many families were sampled, most families contained two and sometimes three MS patients. A number of comparisons have been made using the 162 "healthy supratypes" as controls.

HLA-B7, C4*A3B1, and HLA-DR2 each occur more frequently in the MS supratypes than in non-MS supratypes selected from the same families. Furthermore, the increase in DR2, almost three-fold, is highly significant. These results are consistent with previous reports of associations between B7, and DR2 in particular, and multiple sclerosis. It should be noted, moreover, that these results are likely to be conservative. Supratypes designated MS in these analyses belong to individuals with definite MS. Any founder supratype in a relative but not in a patient was designated non-MS and many of these came from relatives aged 45 years and less who are therefore still at risk for the disease.

Because alleles of the supratype HLA-B7 BF*S C2*1 C4*A3B1 HLA-DR2 were found to occur together more frequently than expected in the total material collected for this study, and because it was the most frequent combination encountered in the MS-family material, it was of interest to determine whether this particular combination occurred more frequently in MS patients. In fact,

approximately 17% of the MS supratypes are B7-S-1-A3B1-2. The same combination occurs approximately one-half as frequently in the controls, and this difference is statistically significant.

Approximately equal proportions of MS and non-MS DR2-supratypes are 7-S-1-A3B1, whereas more MS B7-supratypes are S-1-A3B1-2 than are non-MS B7-supratypes (70% of 44%), and more MS A3B1-supratypes are 7-S-1-2 than are non-MS supratypes (32% of 16%). These results are confusing and not easily interpreted. Most likely they simply reflect (a) the population association between alleles of this supratype, and (b) the highly significantly increased DR2 in the patient group. Since DR2 and A3B1 are positively associated, the effect of the increased DR2 on the A3B1 frequency is an overall increase in the number of A3B1 haplotypes in the patient group. Furthermore, since B7-A3B1-S-1-DR2 is a linkage disequilibrium combination, the effect of the increased DR2 is an increase in the proportion of A3B1 supratypes which are 7-S-1-2. Similarly the effect of the increased DR2 on B7 is an overall increase in B7 and an increase in the proportion of B7 supratypes which are S-1-A3B1-2. Finally, the highly significant association found here between DR2 and MS, and the relatively equal proportions of DR2 supratypes which are 7-S-1-A3B1 in MS and non-MS groups suggest that, if an MHC-linked disease susceptibility gene exists, it is (a) on the HLA-DR side of the complement loci and (b) very close

to DR.

Twenty-four DR2 supratypes in the MS group are non-7-S-1-A3B1, that is, lack one or more of these alleles, as compared to nine in the non-MS group. If there is an MS disease susceptibility gene close to HLA-DR which is frequently found on DR2 supratypes, then one would assume that DR2 non-7-S-1-A3B1 supratypes in patients are more likely to carry the gene. Furthermore, if this susceptibility gene arose on a 7-S-1-A3B1-2 supratype, as evidenced by its higher frequency in patients, and is spreading to other supratypes by recombination, then DR2 non-7-S-1-A3B1 supratypes in patients may be more similar to the founder supratype than DR2 non-7-S-1-A3B1 supratypes in healthy controls. Table V-11 shows the supratypes in the MS and non-MS groups which are DR2 but not 7-S-1-A3B1. There are too few data to reach any definite conclusions. It is interesting to note, however, that eight of 24 MS DR2-supratypes are definite C2*1 C4*A3B1 (i.e. 33%) as compared to one of nine (11%) non-MS DR2-supratypes.

The relationship between MS and putative susceptibility genes is far from clear. One possibility is that there is a single MS disease susceptibility (MSDS) gene which is linked to the MHC very close to DR, and which occurs most commonly on DR2 haplotypes but also, although much less frequently, on others. Possible candidates for other supratypes in the families considered here might

Table V-11: MS and non-MS supratypes which are DR2 but not HLA-B7 BF*S C2*1 C4*A3B1.

MS					non-MS				
HLA-B	BF	C2	C4	HLA-DR	HLA-B	BF	C2	C4	HLA-DR
7	S	1*	A3BQ0	2	7	S	1*	A3BQ0	2
27	S	1	A3BQ0	2	7	S	1*	A3BQ0	2
44	S	1	A3BQ0	2					
7	0?	1*	A3B1	2	44	F	1*	A3B1	2
8	S	1*	A3B1	2					
8	S	1*	A3B1	2					
44	S	1*	A3B1	2					
8	S	1*	A3B1	2					
62	F	1*	A3B1	2					
35	F	1*	A3B1	2					
35	S	1*	A3B1	2					
40	S	1*	A3*B1	2	8	S	1*	A3*B1	2
44	S	1*	A3*B1	2					
8	S	1*	A3*B1	2					
					35	F	1*	A3B1*	2
					35	F	1*	A3*B1*	2
15	S	1*	A4B2	2	62	S	1*	A4B2	2
18	S	1*	A4B2	2	18	S	1*	A4B2	2
18	S	1*	A4B2	2					
18	S	1*	A4B2	2					
18	S	1*	A4B2	2					
8	S	1*	AQOB1	2					
8	S	1*	AQOB1	2					
7	S	1*	AQOB1	2					
45	S	1*	A3*B2	2	7	S	1	A3B2	2
7	S	1	A2B1	2					

include those containing HLA-B27, B37, or B40 (see Table IV-29, p. 160). A second possibility is that there is a single MSDS gene, MHC-linked, but of variable penetrance. The report by Ebers et al. (1982) which indicates that MS sibs are no more likely than expected to share haplotypes, makes these alternatives appear unlikely. A third possibility is that multiple MSDS genes exist, one of which is MHC-linked. In other words, MS families transmit non-MHC MSDS genes against a background which includes an MHC-linked disease susceptibility gene commonly associated with DR2. Perhaps individuals in these families are affected when they inherit the appropriate combination of MHC and non-MHC MSDS genes, or of non-MHC MSDS genes alone.

2. Insulin-dependent diabetes mellitus

Comparison of 28 haplotypes from 14 IDDM patients with (a) 27 haplotypes occurring in their healthy relatives but not in themselves, and (b) 98 haplotypes from healthy, unrelated FS individuals, indicates that the C4 haplotype AQOB1 and the allele C4B3 are increased substantially in these patients.

Associations between HLA-B8, HLA-B15 and diabetes, and more particularly, between HLA-DR3 and HLA-DR4 and diabetes, have previously been reported. The supratypes B8 BF*S C2*1 C3*AQOB1 DR3 and B15 BF*S C2*1 C4*A3B3 DR4 have been shown to be disequilibrium combinations in the total

material considered here. It is likely, therefore, that most of the B8 AQOB1 haplotypes seen in these IDDM patients are DR3 and that at least some of the B3 haplotypes are DR4. Of the four B3 IDDM-supratypes, only two are B15, however, and none were tested for HLA-DR. Furthermore, none of the non-B15 C4*B3-positive haplotypes in the total material used for this study have been tested for DR. C4*AQOB1 and C4*B3 are clearly markers for some "diabetic chromosomes". Whether these C4 associations are secondary to associations between IDDM and DR3 and DR4 remains to be shown.

We have collected C4 phenotype data on 48 unrelated IDDM patients (Skanes et al., in preparation). The C4*B3 allele is found in 13 of these 48 phenotypes as compared to 4/117 randomly-selected controls, giving a relative risk, of 10.5 ($p = .00002$). We could not determine the relative risk for AQOB1 from these population data since the haplotype AQOB1 cannot be deduced with certainty from phenotypes. We have confirmed a previously reported (see Farid and Bear, 1981) increase in HLA-B8 in this IDDM group, however.

Associations between C4 allotypes and IDDM have been reported by others. Awdeh et al. (1980b) found a two-fold increase in C4*foS (AQOB1) in IDDM patients as compared to controls. Bertrams et al. (1981a) have found a rare C4*B4 in 26% of 216 IDDM patients but in none of 100 healthy

controls. Kay et al. (1983) have observed the variant C4*B2.9 in five of eight B15-positive IDDM patients. Because the two variants B4 and B2.9 appear similar electrophoretically to the B3 observed here, and because in all three there is association with B15 DR4, it may be concluded that these studies are identifying the same C4 allotype.

There have been reports of associations between IDDM and rare complement variants other than C4*AQOB1 and C4*B3. Significant increases in BF*F1 and C2*2 have been reported (see Introduction, p. 58). There are also indications that not one, but both haplotypes of the patient are somehow important. Individuals with DR3 and DR4 are reportedly at greater risk than those with DR3 or DR4 alone (Bertrams et al., 1981b; Cudworth and Wolf, 1982). In addition, Dawkins et al. (1983) report that two of five B15 C4*B3-positive IDDM patients also possess the supratype B18 BF*F1 C4*A3BQO DR3 or DR4.

The C4 genotypes of the 14 IDDM patients considered in this study are given in Table V-12. It can be seen that eleven have either AQOB1, A3B3, or A3*B3. One of these eleven patients has AQOB1 and A3B3 together. The second haplotype of the other ten is A3BQO in three cases, and in two cases a null allele cannot be excluded at the A locus. The second haplotype is definitely A3B1 in only three of ten genotypes. In view of the relatively low population

Table V-12: C4 genotypes of 14 IDDM patients.

Number of individuals with:	Second haplotype							
	AQOB1	A3B3	A3BQO	A3B1	A3*B1	A3B1*	A4B2	Total
AQOB1		1	2	3		1	1	8
A3B3 or A3*B3	1		1		2			4
A3B1*						1		1
A3B1				1				1
A3*B2					1			1
Total	1	1	3	4	3	2	1	15(1)

(1) Includes one individual with the genotype A3B3/AQOB1 who was entered twice.

frequency reported for A3BQ0 (9%, see p. 207) relative to A3B1 (55%), this result is interesting. It suggests, although the sample is admittedly small, that a second haplotype, containing A3BQ0, for example, may also be important in these patients. The B18 BF*F1 supratype usually contains A3BQ0. The three A3BQ0 haplotypes occurring in these patients were BF*S C2*1*, however, and none of the total 28 IDDM haplotypes was B18.

Unusual complement variants are implicated in an increasing number of diseases. The C4AQ0 allele is increased in SLE and this increase is greater than that of HLA-B8 (Feilder et al., 1983). C4*B3 is increased in rheumatoid arthritis patients (Dawkins et al., 1983). The associations between C4*AQOB1, C4*B3, BF*F1, C2*2, and IDDM have already been discussed. We have further observed an association between C4*B3 and Graves' disease (Skanes et al., in preparation). The B3 variant has been observed in 16.4% of 61 Graves' disease patients as compared to 3.4% of 117 controls.

There are at least two interpretations of these observations. One is that the complement associations are secondary to strong associations between these diseases and HLA-DR alleles. The implication here is that there are important disease susceptibility genes and one of an unknown number of them is closely linked to the HLA-DR locus. If some chromosomes are hypermutable, as the clustering of

rare complement variants on some chromosomes suggests, then these chromosomes may also contain mutant immune response genes which produce disease susceptibilities.

An alternate hypothesis is that these unusual complement variants play a direct role in the pathology of the disorders in question, a possibility which is supported by the fact that these diseases, IDDM, SLE, rheumatoid arthritis, and Graves' disease, each have an autoimmune component and a pathology in which the formation and disposal of immune complexes is undoubtedly important.

D. Levels of serum C4

Serum C4 concentrations were determined (i) repeatedly over a 6-month period on a panel of 14 healthy volunteers, and (ii) on a single occasion on the stored MS family collection, in order to investigate the sources of variation in serum C4 concentrations; in particular, the effects of handling and storage on samples, and the effects of age, sex, disease states, and gene dose on individual levels.

1. Control panel

C4 levels in healthy individuals are relatively constant over time. The greatest source of variation detected in this study is between individuals (56%), certain

individuals being consistently high (>70mg%), others consistently low (<25mg%), and most having intermediate levels of approximately 30 - 60 mg%.

One of the 14 panel members, HG, had a C4 concentration in the range of 90 - 110mg%. This individual was genotyped C4 A2B2B1*/A2B2B1*, HLA-B14/?w41. Three of his family members were also tested. An MHC-identical brother also had extremely high serum C4. A sister, C4 A3B1/A3B1, had approximately 40mg%, and the mother, C4 A3B1/A2B2B1*, had approximately 60mg%. The C4 electrophoretic patterns of HG, his brother, and mother were similar to that found in other individuals possessing the putative "double B" haplotype A2B2B1. Furthermore, this haplotype is strongly associated with HLA-B14. Because HG and his brother have B14, it is likely that both have at least one, and possible two, A2B2B1 haplotypes. In other words, they are likely to possess five or possibly six C4 structural genes. Since the father of this family is deceased, the second haplotype cannot be conclusively demonstrated.

Four members of the control panel missed work and/or a sampling day during the course of the sampling period because of minor illnesses like influenza or streptococcal infection. All four showed moderate increases in serum C4 levels on the next sampling day, suggesting that the level of C4 protein is, as has been reported, influenced by viral and bacterial infections.

No significant alterations in C4 concentration were apparent in samples from different individuals stored up to 47 weeks at -70° C. There are small differences in mean C4 values obtained in the July reading (lower) and the October reading (higher). It was necessary to use different batches of standard serum for each of the sets of determinations on stored samples. It seems likely that these small differences are due to systematic technical error, introduced either by differences in concentration of the standard sera, or a small dilution error which may have altered the standard reference curve. Technical error is likely to account for some of the residual variance seen in the analysis shown in Table IV-40 (p. 181).

No effect of handling of the samples could be demonstrated. Samples frozen and thawed fifteen times in a two-day period gave values not significantly different from those obtained from the same samples tested within one hour of being drawn. All samples, both the controls and those from the MS families (see below) were handled in the same way. They were thawed in a 37° waterbath, kept over crushed ice, and refrozen quickly at -70° C.

2. Sample from the MS families

All samples from the MS families were tested within a 5-day period, using the same batch and dilutions of standards. A known control was included on every second plate, i.e. 31 times, and values obtained ranged from 21.8-

25.8 mg%, which is within the range of error (±15%) acceptable for the method.

No effect of age, sex, or multiple sclerosis was demonstrated when either all unrelated (two founder haplotypes) or partially-related (one founder haplotype) individuals were considered. The average age of the group was high (approximately 58 years), and the youngest was age 17, suggesting that, in adults, C4 concentration is unrelated to age.

When unrelated individuals with known, definite C4 haplotypes were categorized according to their possession of two, three, or four structural C4 genes, a significant relationship was demonstrated between C4 concentration and the number of C4 genes. There is, in other words, an effect of gene dose on serum C4 concentration.

The variable, "number of genes", accounts for 14-16% of the total variance observed in the analyses shown in Tables IV-43 and IV-44 (pp. 189, 190). This means that, from a knowledge of the number of genes an individual possesses, the C4 concentration can be predicted with about 15% accuracy, or alternatively, that, given C4 concentration, the number of C4 structural genes can be predicted approximately one-seventh of the time. Therefore, although there is a statistically apparent effect of gene dose, C4 serum concentration appears to be of little value in establishing whether an individual has one, two, or no null

alleles. At best, it can occasionally confirm pedigree data.

MHC-identical and MHC-different sib-pairs show the same mean difference in serum C4 levels. This result is surprising in view of the fact that gene dose does, to some extent, affect C4 concentration. It implies that other factors are also important in determining serum C4 concentration, since MHC-identical siblings have inherited the same number and kind of C4 genes.

Clearly, some of the factors are non-genetic. No data were available on the health states other than MS of the individuals compared. Furthermore, since the individuals included in the comparison were all adults, the sibling pairs do not necessarily share a common environment.

C4-high and C4-low strains have been demonstrated in mice. This trait is controlled by genes of the S region of the murine MHC (see Shreffler, 1976). Genetic factors other than C4 structural genes alone may also affect C4 levels in man. If this is the case, then the sib-pair comparison reported here suggests a different control mechanism to that found for C4-high and C4-low mouse strains, and that non-MHC genes may be important. It is tempting to speculate that regulator genes unlinked to the MHC may control either the rates of C4 synthesis and/or catabolism or, alternatively, the number of C4-producing

cells an individual possesses.

E. Summary

The data of this thesis clearly confirm the superiority of C4-typing serum samples after neuraminidase treatment compared with the earlier use of untreated samples. In particular, several gene products with electrophoretic mobilities between F and S were better distinguished by the newer method and, overall, a larger number of alleles was identified.

Generally speaking, the results were compatible with the currently-accepted model for C4 which holds (1) that there are two loci, C4*A and C4*B, per chromosome, (2) that null alleles, AQO and BQO, are relatively common, (3) that C4*A products carry the Rodgers antigenic determinants and C4*B products carry Chido, and (4) that C4*B products are haemolytically active after neuraminidase treatment whereas C4*A products are not. However, a number of families showed haplotypic patterns which did not conform to one or more of these characteristics. First, a few rare gene products were assigned to the A or B series of alleles based on haemolytic activity and on the interpretation of pedigrees assuming two loci per chromosome. The appropriate Rg or Ch antigen could not be demonstrated at all for some of these rare variants, while in others there was evidence of weak or partial inhibition of Rg or Ch antisera. Second, there

were C4 haplotypes which showed A3A2 or B2B1 electrophoretic patterns and, although two separate gene products have not been directly demonstrated, each of these haplotypes appears to contain duplicated A or B loci. If this is the case, then one of these haplotypes, A2B2B1, is likely to contain three C4 structural genes. Furthermore, null alleles, that is, nonfunctioning structural genes, are purely hypothetical. Haplotypes like AQOB1 and A3BQO might just as easily contain a single gene each. These observations support the suggestion that the number of C4 genes is a polymorphism in humans. In other words, the possibility of one, two, and three genes per chromosome, and hence the possibility of two, four, and six genes per genotype, cannot be excluded.

It has been suggested by others that the relative quantity of C4A and C4B antigen in an individual serum sample (as measured by crossed immunoelectrophoresis) indicates the relative number of A and B genes an individual expresses, and that the ratio of A:B antigen can therefore be used in conjunction with phenotypic patterns to determine the individual's genotype. Results obtained in this thesis suggest that this is overly optimistic. While there is likely to be a moderate effect of A and B gene dose within an individual, C4 A:B ratios, in this study at least, are highly variable and of little value in predicting genotype. A large number of individuals could not therefore be completely genotyped because, even with

pedigree and quantitative data, one or more null alleles could not be assigned or excluded with certainty.

Total serum C4 levels are also highly variable. Quantitative serum C4 determinations (by single radial immunodiffusion) from a panel of healthy donors showed that, while the concentration of samples is affected little by long-term storage at -70° C or by repeated freezing and thawing, there is considerable and consistent variation among individuals and that individual levels, although moderately stable over time, are affected by bacterial and viral infections. Furthermore, analysis of serum C4 concentrations of a group of unrelated, C4-typed individuals from multiple sclerosis families revealed no demonstrable effect of age, sex, or multiple sclerosis. Only the number of genes an individual possesses, as determined by C4-typing and pedigree data, was a statistically significant factor in determining serum levels. Because this variable, gene dose, contributed only 15% of the total variance, it is concluded that knowledge of serum C4 concentration is of doubtful value in assigning null alleles to C4 phenotypes. In other words, neither the relative amounts of C4*A and C4*B gene products within an individual, as measured by crossed immunoelectrophoresis, nor the total serum C4 concentration relative to other individuals, as measured by single radial immunodiffusion, can be used to predict accurately the number of C4-structural genes in an individual. Finally, the observation that MHC-identical

siblings were no more likely than MHC-different siblings to have similar serum C4 concentrations suggests that factors other than C4 structural genes alone, or of MHC structural genes alone, are contributing to individual variation in serum C4 levels.

Because of this uncertainty about gene numbers, gene and haplotype frequencies could not be calculated with complete accuracy. Even so, it is apparent that one haplotype, C4*A3B1, is exceedingly common in this material and that four others, AQOB1, A3BQO, A4B2, and A6B1 occur with frequencies ranging from approximately 2 - 12%. No recombination was observed between alleles at the A and B loci and many A-B allele pairs show strong linkage disequilibria.

Analyses of non-random pairwise associations between MHC alleles and of relative deltas for three-, four-, and five-way MHC allelic combinations indicated that certain pairwise combinations of MHC alleles, certain C4-BF-C2 complotypes, and a number of extended MHC supratypes also occur more frequently in this material than expected. In general, the particular combinations observed confirm those described by others, but some are reported here for the first time. For example, the putative homoduplicated haplotypes, A3A2BQO and A2B2B1, were nearly always found with HLA-B14 BF*S C2*1 and HLA-B35 BF*F C2*1 respectively.

MHC recombinant data, analyses of supratype

composition, and comparisons of the relative strengths of associations among pairs within particular supratypes have been used to speculate on the relative position of the C4 loci and on the evolution of MHC supratypes. The data suggest that the C4 loci are between HLA-B and HLA-DR, closer to DR than to B. The difficulty in establishing a clear line of descent for obviously related supratypes suggests that mechanisms other than classical mutation and crossing over, possibly gene conversion and/or suppressed recombination, may have been important in supratype evolution. Furthermore, the frequent clustering of rare, or moderately rare, complement alleles in the same supratype suggests that some complotypes may occupy hypermutable regions of chromosome six.

A sizable number of individuals who were C4-typed for this investigation were multiple sclerosis (MS) and insulin-dependent diabetes (IDDM) patients and their relatives. The MS results show (1) that HLA-B7, C4*A3B1 and, more particularly, HLA-DR2 occur more frequently in "patient" supratypes than in "healthy" supratypes from the same families, and (2) that the supratype B7 BF*S C2*1 C4*A3B1 DR2 is increased in patients. The latter is likely to be an effect of the highly significant association between DR2 and MS, suggesting that one of an unknown number of MS disease susceptibility genes is very closely linked to HLA-DR. High-risk haplotypes, namely C4*AQOB1 and C4*A3B3, were also demonstrated for IDDM. The associations

between these haplotypes and IDDM may simply reflect known associations between IDDM and DR3 and DR4 and, as with MS, suggest DR-associated susceptibility genes. Because other rare complement variants have also been observed in IDDM, it may be that these variants mark hypermutable supratypes containing mutant immune response genes. On the other hand, because the same C4 variants, AQOB1 and A3B3, have been observed in other diseases, systemic lupus erythematosus and Graves' disease for example, it is also possible that these C4 variants play a direct role in certain diseases, particularly those in which immune complex formation and complement activation are likely to be important.

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APPENDIX A: Frequency tables used to calculate standard delta values and non-random associations among various HLA-B, BF, C2, HLA-DR alleles and C4 haplotypes.

Table A-1 : Distribution of BF alleles by C2.

C2	BF				
	S	S1	F	F1	Total
1	668	8	149	4	829
2	37	0	0	0	37
Total	705	8	149	4	866

Table A-2: Distribution of BF alleles by HLA-B.

HLA-B	BF				
	S	S1	F	F1	Total
-	22	0	4	0	26
5	27	0	5	0	32
7	158	0	4	0	162
8	135	0	2	0	137
12	75	4	71	0	150
13	8	0	0	0	8
14	30	0	4	1	35
15	48	0	15	0	63
16	23	0	5	0	28
17	25	1	1	0	27
18	37	0	3	5	45
21	10	2	2	0	14
22	22	0	2	0	24
27	33	2	2	0	37
35	44	0	35	0	79
37	7	0	5	0	12
40	69	0	5	0	74
41	6	0	2	0	8
42	3	1	1	0	5
Total	782	10	168	6	966

Table A-3: Distribution of C2 alleles by HLA-B.

HLA-B	C2		
	1 or 1*	2	Total
-	7	1	8
5	48	1	49
7	158	3	161
8	120	0	120
12	133	5	138
13	7	0	7
14	35	0	35
15	47	8	55
16	25	3	28
17	23	1	24
18	38	0	38
21	11	0	11
22	9	13	22
27	33	0	33
35	73	3	76
37	9	0	9
40	67	2	69
41	9	0	9
42	5	0	5
Total	857	40	897

Table A-4: Distribution of HLA-DR alleles by HLA-B

HLA-B	HLA-DR									
-	1	2	3	4	5	6	7	8	9	Total
-	0	0	0	0	1	1	1	0	0	0	3
5	2	5	1	0	8	2	0	0	0	0	18
7	4	3	49	4	6	3	1	3	0	0	73
8	2	1	5	45	2	0	1	1	0	0	57
12	1	3	4	1	7	1	2	13	0	0	32
13	1	0	0	0	0	0	0	1	0	0	2
14	1	5	0	5	0	2	0	9	0	0	22
15	1	1	4	2	10	1	0	1	0	0	20
16	0	2	0	3	3	2	0	0	0	0	10
17	1	0	2	0	0	1	0	11	0	0	15
18	1	2	5	3	0	0	0	1	0	0	12
21	2	0	0	0	3	1	0	2	0	0	8
22	3	2	2	2	2	0	0	0	0	0	11
27	5	1	4	0	5	1	0	1	0	0	17
35	3	12	4	1	4	5	0	3	0	0	32
37	0	0	1	0	0	1	0	0	0	0	2
40	4	1	2	0	7	4	0	0	1	0	19
41	0	0	1	0	2	1	1	1	0	0	6
42	1	0	1	1	0	0	0	0	0	0	3
Total	31	38	85	67	60	26	6	47	1	0	362

Table A-5 : Distribution of HLA-DR alleles by BF.

	HLA-DR										
BF	-	1	2	3	4	5	6	7	8	9	Total
S	26	26	72	56	61	26	0	25	1	1	294
S1	0	3	0	0	0	0	0	1	0	0	4
F	3	15	5	4	5	4	2	16	0	0	54
F1	0	0	0	1	0	0	0	0	0	0	1
Total	29	44	77	61	66	30	2	42	1	1	353

Table A-6: Distribution of HLA-DR alleles by C2.

	HLA-DR										
C2	-	1	2	3	4	5	6	7	8	9	Total
1	25	37	76	56	55	28	5	39	1	1	323
2	4	0	1	1	7	1	0	0	0	0	14
Total	29	37	77	57	62	29	5	39	1	1	337

Table A-7: Distribution of C4 haplotypes by C2 using complete C4 haplotypes only.

C4	C2		
	1 or 1*	2	Total
AQOB1	101	0	101
AQOB2	8	0	8
AQOB3	1	0	1
A2BQ0	5	0	5
A2B1	8	0	8
A2B2	9	0	9
A2B3	1	0	1
A3BQ0	76	0	76
A3B1	211	2	213
A3B2	2	0	2
A3B3	7	0	7
A4B2	40	22	62
A4B4	1	11	12
A6B1	22	0	22
Total	492	35	527

Table A-8: Distribution of C4 haplotypes by BF using complete C4 haplotypes only.

C4	BF				
	S	S1	F	F1	Total
AQ0B1	111	1	4	0	116
AQ0B2	9	0	0	0	9
AQ0B3	0	0	1	0	1
A2BQ0	1	0	3	0	4
A2B1	4	4	0	0	8
A2B2	8	0	1	0	9
A2B3	0	1	0	0	1
A3BQ0	49	0	33	5	87
A3B1	187	0	42	0	229
A3B2	2	0	0	0	2
A3B3	6	0	1	0	7
A4B2	71	0	1	0	72
A4B4	15	0	0	0	15
A6B1	23	0	1	0	24
Total	486	6	87	5	584

Table A-9: Distribution of C4 haplotypes by HLA-B using complete C4 haplotypes only.

C4	HLA-B																			
	5	7	8	12	13	14	15	16	17	18	21	22	27	35	37	40	41	42	-	Total
AQOB1	0	3	92	3	1	2	1	3	3	1	4	0	0	2	0	1	1	0	0	117
AQOB2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	1	0	0	9
AQOB3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
A2BQO	3	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	6
A2B1	0	1	0	2	0	0	0	2	0	0	0	0	1	0	0	0	0	1	0	7
A2B2	0	0	0	0	0	5	0	1	0	1	1	0	0	0	0	0	0	0	0	8
A2B3	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	2
A3BQO	4	11	2	36	0	0	4	3	0	11	0	2	1	11	0	3	1	0	0	89
A3B1	16	71	13	35	2	5	12	2	2	8	4	0	10	17	3	22	4	3	3	232
A3B3	0	0	0	0	0	0	6	0	0	1	0	1	0	0	0	0	0	0	0	8
A3B2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2
A4B2	5	7	1	7	0	0	12	2	1	12	0	3	6	6	1	7	0	0	1	71
A4B4	0	2	0	0	0	0	0	0	0	0	0	12	0	0	0	1	0	0	0	15
A6B1	1	1	0	1	0	0	1	0	18	0	0	0	1	1	1	0	0	0	0	25
Total	30	97	108	85	3	12	37	14	25	34	9	18	20	37	5	43	7	4	4	592

Table A-10: Distribution of C4 haplotypes by HLA-DR using complete C4 haplotypes only.

C4	HLA-DR										Total
	1	2	3	4	5	6	7	8	9	-	
AQOB1	2	2	41	3	0	1	2	0	0	1	52
AQOB2	1	0	0	2	0	0	0	0	0	1	4
AQOB3	0	0	0	0	0	0	0	0	0	0	0
A2BQO	0	0	0	1	1	0	0	0	0	1	3
A2B1	0	1	2	0	0	0	0	0	0	0	3
A2B2	4	0	0	0	1	0	0	0	0	0	5
A2B3	0	0	0	0	0	0	0	0	0	1	1
A3BQO	3	5	4	8	1	1	1	0	0	2	25
A3B1	5	46	5	17	12	1	14	1	0	8	109
A3B2	0	0	0	0	0	0	0	0	0	0	0
A3B3	0	0	0	3	0	0	0	0	0	0	3
A4B2	2	9	0	14	1	0	1	0	0	5	32
A4B4	0	0	1	1	2	0	0	0	0	3	7
A6B1	0	0	0	1	0	0	10	0	0	0	11
Total	17	63	53	50	18	3	28	1	0	22	255

Table A-11: Distribution of C4 haplotypes by BF using all complete and incomplete C4 haplotypes.

C4	BF				
	S	S1	F	F1	Total
AQOB1	111	1	4	0	116
AQOB2	9	0	0	0	9
AQOB3	0	0	1	0	1
A2BQO	1	0	3	0	4
A2B1	4	4	0	0	8
A2B2	8	0	1	0	9
A2B3	0	1	0	0	1
A3BQO	49	0	33	5	87
A3B1	187	0	42	0	229
A3B2	2	0	0	0	2
A3B3	6	0	1	0	7
A4B2	71	0	1	0	72
A4B4	15	0	0	0	15
A6B1	23	0	1	0	24
A3A2BQO	0	0	3	0	3
A3*A2BQO	1	0	9	0	10
A3A2*BQO	2	0	3	0	5
A3*A2B1*	0	0	4	0	4
A3*A2B2*	0	0	1	0	1
A2B1*	4	5	3	0	12
A2B2*	1	0	0	0	1
A2*B1	2	0	0	0	2
A3*B1	74	0	10	0	84
A3*B1*	121	0	23	1	145
A3B1*	62	0	28	0	90
A3*B2	10	0	0	0	10
A3*B3	8	0	1	0	9
A3B3*	1	0	0	0	1
A4*B2*	1	0	0	0	1
A2B2B1*	7	0	0	0	7
A2B2*B1	3	0	0	0	3
A3*B2B1*	1	0	0	0	1
Total	784	11	172	6	973

Table A-12: Distribution of C4 haplotypes by C2 using all complete and incomplete C4 haplotypes.

C4	C2		
	1 or 1*	2	Total
AQOB1	101	0	101
AQOB2	8	0	8
AQOB3	1	0	1
A2BQO	5	0	5
A2B1	8	0	8
A2B2	9	0	9
A2B3	1	0	1
A3BQO	76	0	76
A3B1	211	2	213
A3B2	2	0	2
A3B3	7	0	7
A4B2	40	26	66
A4B4	1	11	12
A6B1	22	0	22
A3A2BQO	3	0	3
A3*A2BQO	8	0	8
A3A2*BQO	4	0	4
A3*A2B1*	5	0	5
A3*A2B2*	1	0	1
A2B1*	9	0	9
A2B2*	1	0	1
A2*B1	2	0	2
A3*B1	82	0	82
A3B1*	85	0	85
A3*B1*	143	2	145
A3*B2	6	1	7
A3*B3	9	0	9
A3B3*	1	0	1
A4*B2*	1	0	1
A2B2B1*	7	0	7
A2B2*B1	3	0	3
A3*B2B1*	1	0	1
Total	863	42	905

Table A-13: Distribution of C4 haplotypes by HLA-DR using all complete and incomplete C4 haplotypes.

C4	HLA-DR										Total
	1	2	3	4	5	6	7	8	9	-	
AQOB1	2	2	41	3	0	1	2	0	0	1	52
AQOB2	1	0	0	2	0	0	0	0	0	1	4
AQOB3	0	0	0	0	0	0	0	0	0	0	0
A2BQ0	0	0	0	1	1	0	0	0	0	1	3
A2B1	0	1	2	0	0	0	0	0	0	0	3
A2B2	4	0	0	0	1	0	0	0	0	0	5
A2B3	0	0	0	0	0	0	0	0	0	1	1
A3BQ0	3	5	4	8	1	1	1	0	0	2	25
A3B1	5	46	5	17	12	1	14	1	0	8	109
A3B2	0	0	0	0	0	0	0	0	0	0	0
A3B3	0	0	0	3	0	0	0	0	0	0	3
A4B2	2	9	0	14	1	0	1	0	0	5	32
A4B4	0	0	1	1	2	0	0	0	0	3	7
A6B1	0	0	0	1	0	0	10	0	0	0	11
A3A2BQ0	0	0	0	0	0	0	0	0	0	0	0
A3*A2BQ0	5	0	0	1	1	0	0	0	0	0	7
A3A2*BQ0	0	0	0	0	0	0	0	0	0	0	0
A3*A2B1*	2	0	0	0	0	0	0	0	0	0	2
A3*A2B2*	1	0	0	0	0	0	0	0	0	0	1
A2B1*	1	0	0	0	0	0	2	0	0	1	4
A2B2*	0	0	0	0	0	0	0	0	0	1	1
A2*B1	0	0	0	0	0	0	0	0	0	0	0
A3*B1	2	3	4	3	1	1	7	0	0	2	23
A3B1*	5	6	0	5	6	0	6	0	1	2	31
A3*B1*	1	7	4	7	6	2	3	0	0	3	33
A3*B2	1	1	1	1	0	0	0	0	0	0	4
A3*B3	0	0	0	0	0	0	0	0	0	0	0
A3B3*	0	0	0	0	0	0	0	0	0	0	0
A4*B2*	0	0	0	0	0	0	0	0	0	1	1
A2B2B1*	2	0	1	0	1	0	0	0	0	0	4
A2B2*B1	1	0	1	0	0	0	0	0	0	0	2
A3*B2B1*	0	0	0	0	0	0	0	0	0	0	0
Total	38	80	64	67	33	6	46	1	1	32	368

Table A-14: C4 haplotypes by HLA-B using all C4 haplotypes.

C4	HLA-B																			Total
	5	7	8	12	13	14	15	16	17	18	21	22	27	35	37	40	41	42	-	
AQOB1	0	3	92	3	1	2	1	3	3	1	4	0	0	2	0	1	1	0	0	117
AQOB2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	1	0	0	9
AQOB3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
A2BQ0	3	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	6
A2B1	0	1	0	2	0	0	0	2	0	0	0	0	1	0	0	0	0	1	0	7
A2B2	0	0	0	0	0	5	0	1	0	1	1	0	0	0	0	0	0	0	0	8
A2B3	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	2
A3BQ0	4	11	2	36	0	0	4	3	0	11	0	2	1	11	0	3	1	0	0	89
A3B1	16	71	13	35	2	5	12	2	2	8	4	0	10	17	3	22	4	3	3	232
A3B2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2
A3B3	0	0	0	0	0	0	6	0	0	1	0	1	0	0	0	0	0	0	0	8
A4B2	5	7	1	7	0	0	12	2	1	12	0	3	6	6	1	7	0	0	1	71
A4B4	0	2	0	0	0	0	0	0	0	0	0	12	0	0	0	1	0	0	0	15
A6B1	1	1	0	1	0	0	1	0	18	0	0	0	1	1	1	0	0	0	0	25
A3A2BQ0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0	3
A3*A2BQ0	2	0	0	1	0	0	0	1	0	0	0	0	0	6	0	0	0	0	0	10
A3A2*BQ0	0	0	0	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
A3*A2B1*	0	1	0	0	0	0	1	0	0	0	0	0	0	5	0	0	0	0	0	7
A3*A2B2*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
A2B1*	2	0	0	3	0	1	0	1	0	0	1	1	1	0	0	1	0	0	0	11
A2B2*	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
A2*B1	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2
A3*B1	2	17	24	12	2	7	3	2	0	2	0	0	3	10	2	8	0	1	2	97
A3B1*	11	26	0	27	0	3	8	0	0	2	2	0	1	16	0	2	1	0	1	100
A3*B1*	8	35	9	27	2	8	9	6	4	11	1	6	9	11	4	20	1	0	1	172
A3*B2	1	1	0	3	0	0	1	1	0	0	0	0	0	0	0	3	0	0	0	10
A3*B3	0	0	0	1	0	0	8	0	0	0	0	0	0	1	1	1	0	0	0	12
A3B3*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
A4*B2*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
A2B2B1*	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	7
A2B2*B1	0	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	3
A3*B2B1*	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Total	56	178	141	163	8	40	67	29	29	49	14	25	36	89	12	78	9	5	8	1036

Table A-15: Distribution of (a) C4 haplotypes, (b) HLA-B alleles, and (c) BF alleles, obtained by direct count from 960 haplotypes typed for C4, HLA-B and BF.

(a)

C4	No. obs.
A2B1	7
A2B1B2*	3
A2B1*	11
A2B2	9
A2B2B1*	7
A2B2*	1
A2B3	1
A2*B1	2
A2BQ0	6
A3B1	225
A3B1*	88
A3B2	2
A3B3	7
A3B3*	1
A3BQ0	88
A3*B1	84
A3*B1*	141
A3*B2	10
A3*B3	9
A4B2	71
A4B4	15
A6B1	23
AQ0B1	116
AQ0B2	9
AQ0B3	1
A2*A3BQ0	5
A3A2BQ0	3
A3*A2B1*	4
A3*A2B2*	1
A3*A2BQ0	10
	960

(b)

HLA-B	No. obs.
-	9
5	47
7	162
8	137
12	152
13	8
14	35
15	62
16	30
17	27
18	43
21	14
22	24
27	35
35	78
37	12
40	72
41	8
42	5
	960

(c)

BF	No. obs.
S	778
S1	10
F	166
F1	6
	960

Table A-16: Distributions of (a) C4 haplotypes, (b) HLA-B alleles, (c) BF alleles, and (d) C2 alleles, obtained by direct count from 864 haplotypes typed for C4, HLA-B, BF and C2.

(a)

C4	No. obs.
A2B1	7
A2B1B2*	3
A2B1*	8
A2B2	9
A2B2B1*	7
A2B2*	1
A2B3	0
A2*B1	2
A2BQO	6
A3B1	207
A3B1*	77
A3B2	2
A3B3	7
A3B3*	1
A3BQO	75
A3*B1	76
A3*B1*	133
A3*B2	7
A3*B3	8
A4B2	67
A4B4	12
A6B1	20
AQOB1	101
AQOB2	8
AQOB3	1
A2*A3BQO	4
A3A2BQO	3
A3*A2B1*	3
A3*A2B2*	1
A3*A2BQO	8
	864

(b)

HLA-B.	No. obs.
–	9
5	42
7	150
8	142
12	113
13	7
14	34
15	52
16	28
17	23
18	37
21	11
22	21
27	32
35	74
37	10
40	66
41	8
42	5
	864

(c)

BF	No. obs.
S	704
S1	6
F	150
F1	4
	864

(d)

C2	No. obs.
1	824
2	39
0	1
	864

Table A-17: Distributions of (a) C4 haplotypes, (b) HLA-B alleles, (c) BF alleles, (d) C2 alleles, and (e) HLA-DR alleles obtained by direct count from 337 haplotypes typed for C4, HLA-B, BF, C2, and HLA-DR.

(a). C4	No. obs.
A2B1	3
A2B1B2*	2
A2B1*	3
A2B2	5
A2B2B1*	3
A2B2*	1
A2B3	0
A2*B1	0
A2BQO	3
A3B1	104
A3B1*	28
A3B2	0
A3B3	3
A3B3*	0
A3BQO	21
A3*B1	23
A3*B1*	28
A3*B2	4
A3*B3	0
A4B2	32
A4B4	6
A6B1	9
AQOB1	46
AQOB2	3
AQOB3	0
A2*A3BQO	0
A3A2BQO	0
A3*A2B1*	2
A3*A2B2*	1
A3*B2BQO	7
	337

(b). HLA-B	No. obs.
5	18
7	69
8	52
12	27
13	2
14	26
15	22
16	10
17	12
18	11
21	3
22	8
27	17
35	30
37	2
40	16
41	6
42	3
-	3
	337

(c). BF	No. obs
S	276
S1	1
F	59
F1	1
	337

(d). C2	No. obs.
1	322
2	14
0	1
	337

(e). HLA-DR	No. obs.
-	30
1	37
2	76
3	57
4	62
5	29
6	5
7	39
8	1
9	1
	337

APPENDIX B: Complete list of founder or entry haplotypes derived from the family material.

```
RUN LD:DTR
Datatrieve-11, DEC Query and Report System
Version: V02.00, 14-JUL-80
Type HELP for help
DTR> SET DICTIONARY VHAP3
DTR> READY HAPS3
DTR> FIND HAPS3 SORTED BY C4, HLA-B
[1060 records found]
DTR> PRINT ALL
```

SOURCE	SAMPLE	FAMILY	HLA A	HLA C	HLA B	BF	C2	C4	HLA DR	RG	CH
FS	3515	253	28	-	5						
FS	3087	249	11	4	5	5	1*				
WP	0144	9	30		5	5					
WP	0146	9	30	2	5	5	1*				
FS	2089	219	25	-	8	5	1*				
FS	3517	253	1	-	8						
WP	0192	15	1		8						
WP	0197	15	1		8		1*				
WP	0197	15	3		18		1*				
WP	0140	9	2		27	5					
WP	0146	9	2		27	5	1*				
WP	0192	15	24		35	5					
MS	0005	58	31	-	7	5	1*	A2 B1	2		
WC	3043		9		12	51	1	A2 B1			
WP	0002	1	2		12	51	1*	A2 B1			
MS	0372	37	26	7	157	5	1*	A2 B1	7		
WP	0102	6	24		16	5	1*	A2 B1			
WC	3327		11		27	51	1*	A2 B1			
MS	0428	85	2	-	38	5	1	A2 B1	3		
MS	0189	13	2	2	42	51	1*	A2 B1	3		
MS	0481	90	30	-	14	5	1*	A2 B1 B2*	3		
MS	0562	49	26	-	14	5	1*	A2 B1 B2*	1		
WP	0459	33	24		38	5	1*	A2 B1 B2*			
WP	0186	14	11		5	5		A2 B1*			
WC	3669		9		12	51		A2 B1*			
WP	0136	8	2		12	51	1*	A2 B1*			
FS	2064	86	3	-	14	5	1*	A2 B1*			
WP	0135	8	26		16	5	1*	A2 B1*			
MS	0460	39	3	-	22	F	1*	A2 B1*	1		
FS	2397	41	11	1	27	51	1*	A2 B1*			
FS	1626	17	2	3	40	5	1*	A2 B1*			
MS	0512	95	29	-	44	F		A2 B1*	7		
FS	3323	246	2	6	50	51	1*	A2 B1*	7		
MS	0493	76	287		507	51	1*	A2 B1*			
MS	0284	125	11	-	51	F	1*	A2 B1*	-		
MV	4262		2		14	5	1*	A2 B2	1		
FS	3302	245	2	-	14	5	1*	A2 B2			
WC	3135		-		14	5	1*	A2 B2			
MS	0136	92	3	-	14	5	1*	A2 B2	1		
MS	0388	70	28	-	14	5	1*	A2 B2	5		
MV	4295		24		15	F	1*	A2 B2	1	P	
MS	0524	127	24	-	18	5	1*	A2 B2	1		
MS	0160	57	24	-	38	5	1*	A2 B2	7	+	
MS	0517	89	2	-	50	5	1*	A2 B2	7		
FS	1666	22	33	-	14	5	1*	A2 B2 B1*			
WC	1240		31		14	5	1*	A2 B2 B1*			
WP	0263	17	28		14	5	1*	A2 B2 B1*			

MS	0283	125	28	-	14	S	1*	A2	B2	B1*	5			
MS	0356	39	3	-	14	S	1*	A2	B2	B1*	1			
MS	0360	39	33	-	14	S	1*	A2	B2	B1*	1			
MS	0481	90	30	-	14	S	1*	A2	B2	B1*	3			
MS	0045	113	1	-	50	S	1*	A2	B2*		-			
MS	0381	74	1	-	17	S1		A2	B3		-		0	
WP	0105	6	1		5	S	1*	A2	BQO					
WP	0460	33	1	-	5		1*	A2	BQO					
MS	0129	19	1	2	27	F	1*	A2	BQO		-		-	
MS	0525	85	24	-	39	F	1*	A2	BQO		4			
MS	0046	31	29	-	44	F	1*	A2	BQO					
FS	1036	61	2	-	51			A2	BQO		5			
WC	1213		3		16	S	1*	A2*	B1					
WC	1343		3		16	S	1*	A2*	B1					
MS	0192	16	26	-	?	S	1*	A3	B1		?			
FS	3577	258	26	-	-	S	1*	A3	B1		6			
WP	0107	6	2		-	S	1*	A3	B1					
MS	0267	52	33	-	-	F	1*	A3	B1		5			
LA	0117		2		5	S	1*	A3	B1					
LA	0160		9		5		1*	A3	B1					
LA	0176		29		5	F		A3	B1					
LA	0181		9	3	5	S	1*	A3	B1		5			
FS	2649	62	2	-	5	S	1	A3	B1					
FS	3412	252	2	-	5	S	1	A3	B1					
WC	3567		32		5	F	1*	A3	B1					
MV	4263		2		7	S	1*	A3	B1		7			
MV	4270		1		7	S	1*	A3	B1		2			
FS	2312	55	3	-	7	S	1*	A3	B1					
FS	1051	25	29	?	7	S	1*	A3	B1					
FS	2256	36	3	-	7	S	1*	A3	B1		2			
FS	1693	220	3	?	7		1*	A3	B1		2			
FS	2374	218	2	-	7	F	1*	A3	B1					
FS	3469	257	10	-	7	S		A3	B1					
FS	3402	252	3	-	7	S	1*	A3	B1					
FS	3571	258	2	-	7	S	1*	A3	B1		2			
FS	3386	251	3	-	7	S	1	A3	B1		2			
FS	3351	245	2	-	7	S	1*	A3	B1					
FS	3155	243	3	-	7	S	1*	A3	B1					
WC	3017		1		7	S	1*	A3	B1					
WC	1348		2		7	S	1*	A3	B1					
WC	3204		2		7	S	1*	A3	B1					
WC	3272		2		7	S	1*	A3	B1					
WC	3038		2		7	S	1*	A3	B1					
WC	3802		2		7	S	1*	A3	B1					
WC	3976		2		7	S		A3	B1					
WC	1004		3		7	S	1*	A3	B1					
WC	5187		3		7	S	1	A3	B1					
WC	3387		11		7	S	1*	A3	B1					
WP	0001	1	24		7	S		A3	B1					
WP	0009	1	24		7	S	1*	A3	B1					
WP	0037	4	24	2	7	S	1	A3	B1					
WP	0197	8	2		7	S	1	A3	B1					
WP	0344	22	2		7	S	1*	A3	B1					
WP	0347	22	2		7	S	1	A3	B1					
WP	0380	25	3	-	7	S	1*	A3	B1					
WP	0392	27	1		7	S	1*	A3	B1					
MS	0001	116	1	-	7	S	1*	A3	B1		2			
MS	0002	116	24	-	7	S	1*	A3	B1		2			
MS	0033	75	2	-	7	S	1*	A3	B1		27			
MS	0055	75	3	-	7	S	1*	A3	B1		2			

POOR PRINT
Epreuve illisible

MS	0115	65	3	-	7	S	1*	A3	B1	2
MS	0118	47	2	-	7	S	1*	A3	B1	2
MS	0134	41	3	-	7	S	1*	A3	B1	2
MS	0142	47	29	-	7	S	1*	A3	B1	2
MS	0167	10	1	-	7	S	1*	A3	B1	2
MS	0183	13	3	-	7	S	1*	A3	B1	2
MS	0193	16	1	-	7	S	1*	A3	B1	2
MS	0208	55	3	-	7	S	1*	A3	B1	3
MS	0229	21	24	-	7		1*	A3	B1	2
MS	0255	52	9	-	7	S	1*	A3	B1	2
MS	0299	34	3	-	7	S	1*	A3	B1	2
MS	0321	43	24	-	7	S	1*	A3	B1	2
MS	0332	61	24	-	7	S	1*	A3	B1	27
MS	0345	61	11	-	7	S	1*	A3	B1	2
MS	0362	36	2	-	7	S	1*	A3	B1	27
MS	0369	68	3	-	7	S	1*	A3	B1	2
MS	0370	37	24	-	7	S	1*	A3	B1	2
MS	0378	37	24	-	7	S	1*	A3	B1	27
MS	0385	49	2	-	7	S	1*	A3	B1	
MS	0412	119	3	-	7	S	1*	A3	B1	2
MS	0431	99	3	-	7	S	1*	A3	B1	2
MS	0432	99	3	-	7	S	1*	A3	B1	2
MS	0436	99	3	-	7	S	1*	A3	B1	2
MS	0460	39	26	2	7	S	1*	A3	B1	5
MS	0495	99	3	-	7	S	1*	A3	B1	2
MS	0516	89	3	-	7	S	1*	A3	B1	7
MS	0528	85	2	-	7	S	1*	A3	B1	2
MS	0528	85	3	-	7	S	1*	A3	B1	2
MS	0543	123	3	-	7	S	1*	A3	B1	-
MS	0551	121	3	-	7	S	1*	A3	B1	2
MS	0555	116	27	-	7	S	1*	A3	B1	2
MS	0563	99	3	-	7	S	1*	A3	B1	1
MS	0566	17	2	-	7	S	1*	A3	B1	2
MS	0579	103	3	-	7	S	1	A3	B1	2
MS	0582	103	2	-	7	S		A3	B1	2
MS	0362	36	2	-	7	S	1*	A3	B1	27
FS	1670	56	1		8	S	1*	A3	B1	47
FS	2225	35	30	-	8	S		A3	B1	
WC	1137		1		8	S	1*	A3	B1	
WP	0245	16	26		8	F	1*	A3	B1	
MS	0059	75	1	-	8	S	1*	A3	B1	7
MS	0134	41	1	-	8	S	1*	A3	B1	7
MS	0216	32	1	-	8	S	1*	A3	B1	
MS	0228	51	28	-	8	S	1*	A3	B1	2
MS	0231	47	1	-	8	S	1*	A3	B1	2
MS	0238	55	3	-	8	S	1*	A3	B1	3
MS	0370	37	1	-	8	S	1*	A3	B1	27
MS	0524	127	1	-	8	S	1*	A3	B1	3
MS	0255	52	1	-	8	S	1*	A3	B1	2
LA	0100		29		12	F	1*	A3	B1	
FS	2020	54	29	1	12	S	1*	A3	B1	
FS	1997	42	28	-	12	F	1	A3	B1	
BA	1435	208	29	-	12	S	1*	A3	B1	
FS	2017	219	2	5	12	S	1*	A3	B1	
FS	1689	19	29	-	12	F	1*	A3	B1	
FS	3088	249	29	-	12	S	1	A3	B1	
FS	3623	249	2	7	12	S		A3	B1	
FS	3351	245	24	5	12	S	1*	A3	B1	
WC	1145		3		12	S	1*	A3	B1	
WC	3402		29		12	S	1	A3	B1	

WC	1206		31		12	S	1*	A3	B1	
WP	0102	6	3		12	S	1*	A3	B1	
WP	0136	8	32		12	S	1	A3	B1	
WP	0148	10	1		12	S	1*	A3	B1	
WP	0180	13	2		12	F		A3	B1	
WP	0255	17	29		12	F	1*	A3	B1	
WP	0369	24	24		12	F	1*	A3	B1	
WP	0379	25	29	-	12	S	1*	A3	B1	
WP	0386	26	2		12	F	1*	A3	B1	
WP	0388	26	32		12	S		A3	B1	
FS	3110	249	30	-	13	S	1*	A3	B1	
WC	3297		1		13	S	1*	A3	B1	
FS	1654	34	32	-	14	S		A3	B1	7
FS	1109	25	32	7	14	S	1*	A3	B1	
MS	0057	75	3	-	14	F	1*	A3	B1	-
MS	0561	123	32	-	14	S	1*	A3	B1	3
MS	0593	19	3	-	14			A3	B1	7
FS	2132	23	2	3	15	S	1	A3	B1	
FS	2319	232	3	-	15	S	1*	A3	B1	
FS	2286	225	2	3	15	F		A3	B1	
FS	3076	248	10	3	15	F		A3	B1	
WC	3445		2		15	S	1*	A3	B1	
WC	3081		11		15	S	2	A3	B1	
WC	3054		11		15	F	1*	A3	B1	
WP	0028	3	2		15	S	1*	A3	B1	
WP	0089	5	2		15	S	1*	A3	B1	
MS	0108	18	3	3	15	F	1	A3	B1	7
MS	0392	70	1	3	15	F	1*	A3	B1	3
MS	0396	70	17	7	17	S	1*	A3	B1	
MS	0560	96	2	-	17	S	1*	A3	B1	5
LA	0177		9		18	S		A3	B1	
FS	3108	249	3	-	18	S	1*	A3	B1	
WC	5028		2		18	S	1*	A3	B1	
WC	3900		10		18	S	1	A3	B1	
WP	0344	22	9		18	S	1	A3	B1	
WP	0393	27	2		18	S	1*	A3	B1	
MS	0128	19	2	-	18	S	1*	A3	B1	5
MS	0453	83	24		18	S	1*	A3	B1	5
LA	0110		28	2	27	S	1*	A3	B1	
BA	1434	208	24	2	27	S	1*	A3	B1	
FS	3102	248	11	2	27	S	1*	A3	B1	
WP	0305	20	2	2	27	S	1	A3	B1	5
MS	0048	31	27	7	27	S	1*	A3	B1	1
MS	0053	106	3		27	S	1*	A3	B1	
MS	0203	23	31	2	27	S	1*	A3	B1	4
MS	0523	127	32	1	27	S	1*	A3	B1	-
MS	0529	85	24	2	27	S	1*	A3	B1	7
MS	0581	103	1	2	27	S	1*	A3	B1	4
MV	4312		28	4	35	F	1*	A3	B1	7
LA	0194		11	4	35	S	1*	A3	B1	
FS	1997	42	28	6	35	S	1	A3	B1	
FS	2975	80	3	4	35	S		A3	B1	
FS	2320	232	2	4	35	F	1*	A3	B1	
FS	3386	251	30	4	35	F	1	A3	B1	7
FS	3385	251	3	4	35	S	1	A3	B1	2
FS	2784	247	24	-	35	S	2	A3	B1	
WC	9001		1		35	F		A3	B1	
WC	3806		3		35	S	1*	A3	B1	
WC	5063		11		35	S	1	A3	B1	
WC	2087		28		35	F	1	A3	B1	

WP	0037	4	2	4	35	S	1	A3	B1		
MS	0224	32	9	4	35	S	1	A3	B1		
MS	0382	74	24	4	35	S	1*	A3	B1	5	
MS	0453	83	2		35	F	1*	A3	B1	2	
FS	3121	240	3	-	37	F		A3	B1		
MS	0072	106	26		37	F	1*	A3	B1		
MS	0164	11	7	-	37	F	1*	A3	B1	2	
FS	3824	258	1	-	377			A3	B1	4	
FS	2240	35	24	-	39	S		A3	B1		
MS	0360	39	2	-	39	S	1*	A3	B1	1	
LA	0117		32	3	40	S	1*	A3	B1		
FS	2373	218	11	3	40	S	1	A3	B1		
FS	3535	257	2		40	S		A3	B1		
FS	2966	247	3	3	40	S		A3	B1		
WC	1235		2		40	S	1*	A3	B1		
WC	1043		2		40	S	1	A3	B1		
WC	3667		2		40	S	1	A3	B1		
WC	3373		2		40	S	1*	A3	B1		
WC	3527		2		40	F	1*	A3	B1		
WC	1056		11		40	S	1	A3	B1		+
WP	0031	3	32		40	S	1*	A3	B1		
WP	0103	6	11		40	F	1*	A3	B1		
MS	0165	11	1	2	40	S	1*	A3	B1	7	
MS	0173	13	31	3	40	S	1*	A3	B1	4	
MS	0182	13	28	3	40	S	1*	A3	B1	4	
MS	0184	13	31	3	40	S	1*	A3	B1	4	
MS	0194	16	2	2	40	S	1*	A3	B1	8	
MS	0341	96	1	2	40	S	1*	A3	B1	5	
MS	0401	73	26	-	40	S	1*	A3	B1	-	
MS	0436	99	2	3	40	S	1*	A3	B1	5	
MS	0546	121	28	3	40	S	1*	A3	B1	4	
MS	0401	73	267	-	407	S	1*	A3	B1	87	
LA	0152		9	2	41	S	1*	A3	B1	2	P
LA	0157		9	3	41	S	1	A3	B1	4	
FS	1996	42	3	-	41	S	1	A3	B1		
WP	0461	33	2		41		1*	A3	B1		
MS	0216	32	30	-	42	S	1	A3	B1		
MS	0374	37	2	-	42	F	1*	A3	B1	-7	
MS	0395	73	29	-	42	S	1*	A3	B1	2	
MS	0046	31	31	-	44	F	1	A3	B1		
MS	0204	23	2	5	44	S	1*	A3	B1	4	
MS	0260	55	1	-	44	S	1*	A3	B1	5	
MS	0271	60	26	-	44	S	1*	A3	B1	-	
MS	0330	61	11	-	44	F	1*	A3	B1	2	
MS	0356	39	29	-	44	S	1*	A3	B1	2	
MS	0364	36	24	-	44	F	1*	A3	B1	7	
MS	0386	70	23	-	44	F		A3	B1		
MS	0425	75	29	-	44	F	1*	A3	B1	7	
MS	0565	17	1	-	44	F	1*	A3	B1	77	
MS	0567	17	29	-	44	F	1*	A3	B1	7	
MS	0571	103	29	-	44	F	1*	A3	B1	7	
MS	0571	103	29	-	44	F	1*	A3	B1	7	
MS	0580	103	2	1	44	S	1	A3	B1	7	
FS	2647	62	30	-	477	S	1*	A3	B1		
MS	0166	11	2	-	49	S	1*	A3	B1	4	
MS	0286	125	23	-	49	S	1*	A3	B1	-	
MS	0460	83	23		49	S	1*	A3	B1	5	
MS	0116	65	2	4	50	F	1*	A3	B1	7	
MV	4271		26		51	S	1*	A3	B1	1	
FS	1035	61	2	-	51	S	1*	A3	B1	4	

MS	0035	113	2	-	51	S	1*	A3	B1	1
MS	0228	21	2	1	51	S	1*	A3	B1	4
MS	0236	55	11	-	51	S	1*	A3	B1	4
MS	0247	29	1	-	51	S	1*	A3	B1	4
MS	0308	70	11	-	51	S	1*	A3	B1	4
MS	0542	123	30	-	51	S	1	A3	B1	4
MS	0291	28	11	-	52			A3	B1	2
WC	1030		28		53	F	1	A3	B1	
MS	0010	58	3	3	60	S	1	A3	B1	4
MS	0317	55	32	3	62	F	1	A3	B1	2
FS	2571	41	-		-	S	1*	A3	B1*	
FS	1765	21	24	7	5	S	1*	A3	B1*	
FS	3491	253	3	2	5	S		A3	B1*	1
FS	3324	246	31	2	5	S	1*	A3	B1*	5
WC	3421		11		5	S	1*	A3	B1*	
WC	3469		32		5	S	1*	A3	B1*	
WP	0013	2	3		5		1*	A3	B1*	
FS	2297	57	3	-	7	S	1	A3	B1*	7
FS	1956	57	3	-	7	S	1*	A3	B1*	2
FS	2571	41	2	-	7	S	1*	A3	B1*	
FS	2400	41	2	-	7	S	1*	A3	B1*	
FS	1766	21	1	-	7	S	1*	A3	B1*	
FS	1631	17	3	-	7	S	1*	A3	B1*	
FS	2975	80	28	-	7			A3	B1*	
FS	2410	219	2	-	7	S	1*	A3	B1*	
FS	1689	19	1	-	7	S	1*	A3	B1*	
FS	3493	253	2	-	7	S		A3	B1*	5
FS	3637	253	3	-	7			A3	B1*	
WC	3321		2		7	S	1*	A3	B1*	
WC	1251		2		7	S		A3	B1*	
WC	3417		2		7	S		A3	B1*	
WC	1250		3		7	S	1*	A3	B1*	
WC	3719		28		7		1*	A3	B1*	
MS	0039	113	3	-	7	S	1*	A3	B1*	7
MS	0040	113	3	-	7	S	1*	A3	B1*	7
MS	0045	113	3	-	7	F	1*	A3	B1*	4
MS	0125	21	1	-	7	S	1*	A3	B1*	1
MS	0273	27	3	-	7	S	1*	A3	B1*	4
MS	0276	20	3	-	7	S	1*	A3	B1*	47
MS	0286	125	3	-	7	S	1*	A3	B1*	2
MS	0442	92	3	-	7	S	1*	A3	B1*	2
MS	0450	88	30	-	7	S	1*	A3	B1*	2
MS	0514	95	2	-	7	S	1*	A3	B1*	2
FS	2632	58	1	5	12	S	1*	A3	B1*	
FS	2311	55	2	5	12	S	1*	A3	B1*	
FS	2312	55	29	-	12	F	1*	A3	B1*	
FS	2257	36	2	-	12	S	1*	A3	B1*	9
FS	1626	17	2	-	12	S	1*	A3	B1*	
FS	2791	63	29	7	12	F	1*	A3	B1*	
FS	1697	220	2	5	12		1*	A3	B1*	7
FS	2005	219	2	-	12	S	1*	A3	B1*	
FS	3725	253	29	-	12	F		A3	B1*	
FS	3638	253	29	-	12			A3	B1*	
FS	3056	247	2	5	12	S	1*	A3	B1*	
WC	3300		2		12	F	1*	A3	B1*	
WC	3465		2		12	F	1*	A3	B1*	
WC	3001		2		12	F	1*	A3	B1*	
WC	2014		2		12	F	1*	A3	B1*	
WC	3050		2		12		1*	A3	B1*	
WC	3615		29		12	F	1*	A3	B1*	

WP	0011	2	1		12	F	1*	A3	B1*	
WP	0082	5	2		12	S		A3	B1*	
WP	0120	7	3		12	F	1*	A3	B1*	
WP	0262	17	9		12	F	1*	A3	B1*	
WC	6309		3		14		1*	A3	B1*	
MS	0005	58	26	-	14			A3	B1*	7
MS	0442	92	30	-	14	S	1	A3	B1*	7
LA	0180		2	3	15	S	1*	A3	B1*	4
LA	0243		2	3	15	S		A3	B1*	
FS	2791	63	30	3	15	S	1*	A3	B1*	
WC	3557		9		15	S	1*	A3	B1*	
WP	0010	2	1		15	F	1*	A3	B1*	
WP	0016	2	1		15	F	1*	A3	B1*	
MS	0386	70	28	3	15	F		A3	B1*	
MS	0491	96	27		157	F	1	A3	B1*	
WP	0121	7	28		18	F	1*	A3	B1*	
WP	0147	10	2		18	S		A3	B1*	
WP	0186	14	24		21	S		A3	B1*	
WP	0263	17	2		21	F	1*	A3	B1*	
WC	3208		3		27	S	1*	A3	B1*	
MV	4210		2	4	35	F	1*	A3	B1*	5
LA	0199		3	4	35	F	1*	A3	B1*	
FS	1654	34	11	4	35	F		A3	B1*	4
FS	1667	22	11	4	35	S	1*	A3	B1*	
FS	2854	66	9	4	35	S	1*	A3	B1*	
FS	2061	86	24	4	35	S	1*	A3	B1*	
WC	3058		2		35		1*	A3	B1*	
WC	3908		3		35	F	1*	A3	B1*	
WP	0436	32	31		35			A3	B1*	
MS	0128	19	24	4	35	S	1*	A3	B1*	-
MS	0144	41	2	4	35	S	1*	A3	B1*	-
MS	0185	13	3	4	35	F	1*	A3	B1*	1
MS	0322	43	11	4	35	S	1*	A3	B1*	5
MS	0443	92	2	4	35	S	1*	A3	B1*	2
MS	0502	16	11	4	35	F	1*	A3	B1*	27
MS	0596	19	9	4	35			A3	B1*	5
FS	1631	17	2	2	40	S	1*	A3	B1*	
FS	3323	246	30	-	41	S	1*	A3	B1*	5
WP	0459	33	3		44	S	1*	A3	B1*	
MS	0073	106	28	-	44	S	1*	A3	B1*	1
MS	0135	41	29	7	44	F	1*	A3	B1*	7
MS	0155	63	3	-	44	S	1*	A3	B1*	87
MS	0155	63	29	-	44	F	1*	A3	B1*	7
MS	0276	20	2	-	44	S	1*	A3	B1*	47
MS	0481	96	247		447	F	1*	A3	B1*	
MV	4330		2		51	S	1	A3	B1*	4
Wp	0483	35	26	4	51	S	1*	A3	B1*	
WP	0483	35	3	-	51	F	1*	A3	B1*	
WP	0483	35	26	4	51	S	1*	A3	B1*	
MS	0341	96	32	2	51	S	1*	A3	B1*	1
MS	0119	47	7	3	60	S	1*	A3	B1*	
MS	0326	68	3	-	62	S	1*	A3	B1*	
MS	0261	55	3	-	7	S	1*	A3	B2	
MS	0047	31	2	3	40	S	1*	A3	B2	
WC	3664		11		35	S	1*	A3	B3*	
FS	2810	66	2	3	15	S	1*	A3	B3~	
FS	2411	219	2	3	15			A3	B3~	
MS	0186	41	1	-	15	S	1*	A3	B3~	47
MS	0378	37	3	3	15	S	1*	A3	B3~	4
MS	0429	99	2	3	15	S	1*	A3	B3~	4

MS	0397	70	3	-	18	F	1*	A3	B3~				
WP	0168	12	11		22	S	1*	A3	B3~				
MS	0118	47	3	3	62	S	1*	A3	B3~	4			
FS	2065	54	31	1	5	F	1*	A3	BQO				
FS	2286	225	3	2	5	S		A3	BQO				
FS	3156	243	2	-	5	S	1*	A3	BQO				
FS	3468	257	2		7	S		A3	BQO				
WC	1083		3		7	S	1*	A3	BQO			0	
WC	1042		3		7	S	1*	A3	BQO			0	
WC	3312		32		7		1*	A3	BQO			0	
WP	0092	5	2		7	F	1*	A3	BQO				
WP	0099	5	1		7	F	1*	A3	BQO				
MS	0129	19	24	-	7	S	1	A3	BQO	2	-		
MS	0167	10	2	-	7	S	1*	A3	BQO	2			
MS	0186	41	3	-	7	S	1*	A3	BQO	47			
MS	0192	16	2	-	7	S	1*	A3	BQO	2			
MS	0259	52	2	-	7	S	1*	A3	BQO	4			
WC	3726		2		8	S	1	A3	BQO				
WP	0434	31	1		8	S	1*	A3	BQO				
FS	2633	58	29	-	12	F	1*	A3	BQO				
BA	1420	61	33	5	12	S	1*	A3	BQO	7			
FS	2421	39	2	7	12	F		A3	BQO			0	
FS	2387	39	2	7	12	S		A3	BQO			0	
FS	3482	253	2	5	12	S		A3	BQO	4			
FS	3479	253	31	5	12	F		A3	BQO	67			
FS	3577	258	11	-	12	F	1*	A3	BQO	6			
FS	3174	249	2	5	12	S	1*	A3	BQO				
FS	3156	243	2	5	12	S	1*	A3	BQO				
FS	3102	248	2	5	12	F	1*	A3	BQO				
FS	3105	248	29	-	12	S	1*	A3	BQO				
WC	1040		2		12	F	1	A3	BQO			0	
WC	1170		2		12	F	1*	A3	BQO			0	
WC	1207		2		12	F	1*	A3	BQO			0	
WC	5097		2		12	F	1*	A3	BQO			0	
WC	5097		2		12	F	1*	A3	BQO			0	
WC	3829		2		12	F	1*	A3	BQO			0	
WC	1254		2		12	S	1*	A3	BQO				
WC	1170		30		12	S	1	A3	BQO			0	
WC	2047		31		12	S	1*	A3	BQO				
WC	1206		31		12	S	1	A3	BQO				
WP	0036	4	2	-	12	S	1	A3	BQO				
WP	0393	27	2		12	S	1*	A3	BQO				
WP	0434	31	3		12	S	1*	A3	BQO				
LA	0168		11	3	15	S		A3	BQO				
FS	2065	54	2	5	15	F	1*	A3	BQO				
WP	0168	12	2		15	S	1*	A3	BQO				
MS	0381	74	2	3	15	F		A3	BQO	3			
LA	0195		11	5	18	F1	1*	A3	BQO		P		
FS	2421	39	30	7	18	F1		A3	BQO			0	
FS	3116	248	25	-	18	S		A3	BQO				
WC	2006		10		18	S	1	A3	BQO			0	
WC	1083		25		18	S	1	A3	BQO			0	
WC	2194		28		18	F1	1	A3	BQO		P	0	
WC	3352		30		18	F1		A3	BQO			0	
WC	3829		30		18	S	1	A3	BQO			0	
MS	0247	29	2	-	18	S	1*	A3	BQO	-			
MS	0517	89	25	-	18	F	1*	A3	BQO	7			
MS	0520	127	30	-	18	F1	1*	A3	BQO	7			
WP	0109	6	10		22	S	1*	A3	BQO				
MS	0502	103	24	3	22	S		A3	BQO	3			

MS	0292	23	3	2	27	S	1	A3	BQ0	2				
MV	4305		3	4	35	F	1*	A3	BQ0	1	*	O		
MV	4306		3	4	35	F	1*	A3	BQ0	1	*	O		
FS	3414	252	11	4	35	F	1	A3	BQ0					
FS	3174	249	2	4	35	S	1*	A3	BQ0					
WC	3758		2		35	F	1*	A3	BQ0			O		
WC	1103		3		35	F	1*	A3	BQ0					
WC	1051		11		35	S	1*	A3	BQ0			O		
WC	1011		28		35	F	1	A3	BQ0			O		
WP	0433	31	26		35	S		A3	BQ0					
MS	0226	13	3	4	35	F	1*	A3	BQ0	1		O		
MS	0468	83	30		35	S	1*	A3	BQ0	-				
MS	0523	127	2	3	38	F	1*	A3	BQ0	3				
FS	1035	61	24	-	39	F	1*	A3	BQ0	4				
MS	0382	74	26	-	39	S	1*	A3	BQ0	3				
WP	0105	6	31		40	F	1*	A3	BQ0					
WP	0379	25	11	3	40	S	1*	A3	BQ0					
WP	0460	33	31	3	40		1*	A3	BQ0					
FS	3411	252	30	-	41	S	1	A3	BQ0					
WC	1169		2		44	F	1*	A3	BQ0			O		
WC	1229		2		44	F	1*	A3	BQ0			O		
WC	1229		2		44	F	1*	A3	BQ0			O		
WC	1051		2		44	F	1*	A3	BQ0			O		
MS	0226	13	2	-	44	S	1*	A3	BQ0	4		O		
MS	0243	32	2	-	44	S	1*	A3	BQ0					
MS	0256	29	25	-	44	S	1*	A3	BQ0	2				
MS	0270	60	3	-	44	S	1*	A3	BQ0	4				
MS	0345	61	25	-	44	F	1*	A3	BQ0	7				
MS	0402	73	2	-	44	S	1*	A3	BQ0	4				
MS	0551	121	30	-	44	S	1*	A3	BQ0	4				
MS	0575	103	2	-	44	S	1*	A3	BQ0	4				
MS	0194	16	1	?	51	F	1*	A3	BQ0	5				
MS	0332	61	26	-	-	S	1*	A3*B1		27				
MS	0332	61	26	-	-	S	1*	A3*B1		27				
WP	0121	7	24		5	S	1*	A3*B1						
WP	0247	116	2		5	S	1*	A3*B1						
FS	2256	36	2	-	7	S	1*	A3*B1		-				
DA	1268	104	3	-	7			A3*B1						
DA	1432	207	3	-	7	S		A3*B1						
FS	2964	247	1	-	7	S	1*	A3*B1						
WC	3224		2		7	S	1*	A3*B1						
WC	3203		2		7	S	1*	A3*B1						
WC	3580		2		7	S	1*	A3*B1						
WC	1021		3		7	S	1	A3*B1						
WC	1124		3		7	S	1*	A3*B1						
WC	3584		3		7	S	1*	A3*B1				+		
WP	0090	5	9		7	S	1*	A3*B1						
WP	0435	32	24		7		1*	A3*B1						
MS	0108	18	2	?	7	S	1*	A3*B1		1				
MS	0185	13	2	-	7	S	1*	A3*B1		3				
MS	0312	50	3	-	7	S	1*	A3*B1		4				
MS	0312	50	24	-	7	S	1*	A3*B1		2				
MS	0325	68	3	-	7	S	1*	A3*B1						
MV	4291		1		8	S	1	A3*B1		4				
FS	2311	55	1	-	8	S	1*	A3*B1						
FS	2240	35	2	-	8	S		A3*B1						
FS	1766	21	32	-	8	S	1*	A3*B1						
FS	1697	220	1	-	8		1*	A3*B1		?				
FS	2346	231	2	-	8			A3*B1						
FS	3533	257	1		8	S		A3*B1						

FS	3725	253	1	-	8	S		A3*B1		7
FS	3638	253	1	-	8			A3*B1		
FS	3637	253	1	-	8			A3*B1		
FS	0000	229	1	-	8	S	1*	A3*B1		
FS	0000	229	1	-	8	S	1*	A3*B1		
FS	2783	247	1	-	8	S	1	A3*B1		
BB	1213	241	2	7	8	S	1*	A3*B1		
WC	6302		1		8	S	1*	A3*B1		
WC	1077		1		8	S	1*	A3*B1		
WC	3482		9		8	S	1*	A3*B1		
WP	0120	7	1		8	S	1*	A3*B1		
WP	0242	16	2		8	S	1*	A3*B1		
MS	0039	113	1	-	8	S	1*	A3*B1		-
MS	0219	12	1	7	8	S	1*	A3*B1		3
MS	0325	68	1	-	8	S	1*	A3*B1		
MS	0543	123	1	-	8	S	1*	A3*B1		4
MS	0560	96	24	-	8	S	1*	A3*B1		2
FS	2477	234	25	-	12	F		A3*B1		
FS	3086	249	29	7	12	F	1*	A3*B1		
WC	6305		2		12	S		A3*B1		
WC	3851		9		12		1*	A3*B1		
WC	3274		9		12		1*	A3*B1		
WC	3445		11		12	S	1*	A3*B1		
WP	0090	5	2		12	S	1*	A3*B1		
WP	0169	12	29		12	F	1*	A3*B1		
FS	3088	249	30	5	13	S	1*	A3*B1		
FS	2270	240	28	-	13	S	1*	A3*B1		
MV	4209		3		14	S	1*	A3*B1		7
FS	2965	247	11	-	14	F	1*	A3*B1		
FS	2913	247	11	-	14	S	1	A3*B1		
FS	3267	241	3	-	14	S	1*	A3*B1		
MS	0040	113	26	-	14	S	1*	A3*B1		7
MS	0209	55	2	-	14	F	1*	A3*B1		3
MS	0311	50	2	-	14	S	1*	A3*B1		7
WC	1208		2		15	S	1*	A3*B1		
WC	3325		2		15	S	1	A3*B1		
MS	0163	12	24	3	15	S	1	A3*B1		5
WC	3270		3		16	S	1	A3*B1		
WC	5053		3		16	S	1*	A3*B1		
WC	2199		2		18	S	1*	A3*B1		
WC	2085		32		18	S	1*	A3*B1		
MV	4313		11	2	27	S	1*	A3*B1		2
WC	3849		28		27	S	1*	A3*B1		
WP	0181	13	-		27	S		A3*B1		
BA	1214	207	28	4	35			A3*B1		
WC	3736		1		35	F	1	A3*B1		
WC	5515		2		35		1*	A3*B1		
WC	5515		3		35		1*	A3*B1		
WC	1105		3		35			A3*B1		
WC	5257		11		35	F	1*	A3*B1		
WC	3476		31		35	F	1*	A3*B1		
MS	0053	106	3		35	S	1*	A3*B1		
MS	0073	106	3	4	35	S	1*	A3*B1		7
MS	0249	29	30	-	35	S	1*	A3*B1		3
FS	2387	39	1	7	37	S		A3*B1		
WC	3812		2		37	S	1*	A3*B1		
FS	2344	231	28	3	40		1*	A3*B1		
FS	2478	234	2	-	40	S		A3*B1		
FS	3404	252	28	37	40	S	1*	A3*B1		
WC	6306		2		40	S	1*	A3*B1		

WC	1146		2		40	5	1*	A3*B1		
WC	1503		3		40	5	1*	A3*B1		+
WC	1105		32		40	5	1	A3*B1		
MS	0303	50	3	2	40	5	1*	A3*B1		
WC	5189		3		42	5	1	A3*B1		
MV	4210		24	2	44	5	1*	A3*B1	6	
MV	4330		1	4	44	F	1*	A3*B1	7	
MS	0176	12	29	?	44	F	1*	A3*B1	7	
MS	0290	28	29	-	44			A3*B1	7	
MS	0049	31	11	37	54	5	1*	A3*B1	1	
MS	0498	77	2	?	?	5	1*	A3*B1*	?	
MS	0087		23	-	-	5	1*	A3*B1*		
FS	2133	23	2	-	5	5	1	A3*B1*		
BA	1269	104	19	-	5			A3*B1*		
FS	3823	258	-	-	5			A3*B1*	-	
FS	3236	238	2	2	5	5	1*	A3*B1*		
FS	1827	2	3	-	5	5	1*	A3*B1*		
WP	0018	2	11		5		1*	A3*B1*		
WP	0018	2	2		5		1*	A3*B1*		
WP	0312	19	2		5	F	1*	A3*B1*		
MV	4238		2		7	5	1*	A3*B1*	6	
FS	2650	51	3	-	7	5	1	A3*B1*		
FS	1635	29	2	-	7	5	1*	A3*B1*		
FS	1635	29	9	-	7	5	1*	A3*B1*		
FS	1845	20	3	-	7			A3*B1*		
FS	2132	23	3	-	7	5	1	A3*B1*		
FS	2724	209	3	-	7	5	1*	A3*B1*		
FS	3401	252	2	-	7	5	1*	A3*B1*		
FS	3304	245	3	-	7	5	1*	A3*B1*		
BB	1231	237	24	-	7	5	1*	A3*B1*		
FS	2712	237	2	-	7	5	1*	A3*B1*		
WC	3535		1		7	5	1*	A3*B1*		
WC	3538		1		7	5	1*	A3*B1*		
WC	2036		2		7	5	1*	A3*B1*		
WC	2071		2		7	5	1*	A3*B1*		
WC	7156		3		7		1*	A3*B1*		
WC	5523		3		7	5	1*	A3*B1*		
WC	3165		28		7		1	A3*B1*		
WC	8212		29		7		1*	A3*B1*		
WP	0169	12	2		7	5	1*	A3*B1*		
WP	0181	13	3		7	5		A3*B1*		
WP	0474	34	2	-	7	5	1*	A3*B1*		
WP	0474	34	3	2	7		1*	A3*B1*		
WP	0309	19	3		7	5	1*	A3*B1*		
MS	0049	31	3	-?	7	5	1*	A3*B1*	2	
MS	0078		3		7	5	1*	A3*D1*		
MS	0078		3		7	5	1*	A3*B1*		
MS	0091		2	-	7	5	1*	A3*B1*		
MS	0260	55	3	-	7	5	1*	A3*B1*	-	
MS	0275	20	3	-	7	5	1*	A3*B1*	4	
MS	0303	50	3	-	7	5	1*	A3*B1*		
MS	0355	39	3	-	7	5	1*	A3*B1*	2	
MS	0407	77	1	-	7	5	1*	A3*B1*	2	
MS	0450	88	11	-	7	5	1*	A3*B1*	?	
MS	0499	77	3	-	7	5	1*	A3*B1*	2	
MS	0275	70	24	-	77	5	1*	A3*B1*	2	
MV	4325		1		8	5	1*	A3*B1*	3	
FS	1760	52	1	-	8	5	1*	A3*B1*		
FS	2428	37	1	-	8	5		A3*B1*		
FS	3485	254	1	-	8	5		A3*B1*		

FS	3324	246	25	-	8	S	1*	A3*B1*	3
BB	1231	237	1	-	8	S	1*	A3*B1*	
FS	1825	2	1	-	8	S	1*	A3*B1*	3
WC	3922		1		8	S	1*	A3*B1*	
WC	3028		1		8	S	1*	A3*B1*	
FS	2620	51	2	5	12		1	A3*B1*	
FS	2428	37	25	5	12	F		A3*B1*	
FS	2393	37	2	-	12	F		A3*B1*	
FS	2364	37	25	-	12	F		A3*B1*	
FS	3403	252	24	-	12	F	1*	A3*B1*	
FS	2337	229	11	-	12	S	1*	A3*B1*	
FS	3237	238	2	3	12	S	1*	A3*B1*	
FS	2269	240	3	5	12	S	1*	A3*B1*	
WC	1165		2		12	S	1*	A3*B1*	
WC	1097		2		12	S	1*	A3*B1*	
WC	3913		10		12	S	1*	A3*B1*	
WC	2054		28		12		1*	A3*B1*	
WC	3162		29		12	S	1*	A3*B1*	
WC	3553		29		12	F	1*	A3*B1*	
WC	3913		29		12	F	1*	A3*B1*	
WP	0009	1	3		12	F		A3*B1*	
WP	0016	2	29		12	F	1*	A3*B1*	
WP	0107	6	3		12	F	1*	A3*B1*	
WP	0187	14	25		12	F		A3*B1*	
WP	0438	32	2		12			A3*B1*	
MV	4263		2	6	13	S	1*	A3*B1*	
MS	0451	88	2	-	13	S	1*	A3*B1*	-
FS	1763	52	32	-	14	S	1*	A3*B1*	7
FS	1849	20	32	-	14			A3*B1*	
FS	2269	240	3	-	14	S	1*	A3*B1*	
WP	0375	24	24		14	S	1*	A3*B1*	
WP	0438	32	1		14			A3*B1*	
MS	0416	77	26	-	14	S	1*	A3*B1*	7
MS	0496	77	3	-	14	F1	1*	A3*D1*	3
MS	0501	119	3	-	14	F	1*	A3*D1*	7
MV	4325		2	4	15	S	1*	A3*B1*	4
FS	2712	237	2	3	15	F	1*	A3*B1*	
FS	1827	2	2	5	15	S	1*	A3*B1*	4
WC	3399		3		15	S	1*	A3*B1*	
WC	3480		9		15	S	1*	A3*B1*	
WP	0135	8	24		15	S	1*	A3*B1*	
WP	0247	16	31		15	S	1*	A3*D1*	
WP	0371	24	31		15		1*	A3*B1*	
WP	0475	34	28	3	15	F	1*	A3*B1*	
FS	1845	20	25	-	16			A3*B1*	
WC	2033		1		16	S	1	A3*B1*	
WC	3593		11		16	S	1*	A3*B1*	
FS	1763	52	1	-	17	S	1*	A3*B1*	7
FS	1636	29	1	-	17	F	1*	A3*D1*	
FS	1849	20	1	-	17			A3*B1*	
FS	2724	209	11	-	17	S	1*	A3*B1*	
FS	2934	90	24	?	18	S	1*	A3*B1*	
FS	2727	209	2	-	18	S	1*	A3*D1*	
FS	2344	231	107	-	18			A3*B1*	
FS	3485	254	25	-	18	S		A3*B1*	
WP	0017	2	10		18		1*	A3*B1*	
WP	0365	23	11		18			A3*B1*	
MS	0054	106	2	?	18		1*	A3*D1*	7
MS	0091		25	-	18	S	1*	A3*D1*	
MS	0145	92	11	-	18	S	1*	A3*D1*	1

MS	0146	58	2	-	18	S	1*	A3*B1*	5
MS	0163	12	30	7	18	S	1*	A3*B1*	7
FS	2934	90	11	7	22	S	1*	A3*B1*	
FS	2337	229	1	-	22	F	1*	A3*B1*	
FS	3237	238	11	3	22	S	1*	A3*B1*	
WC	3125		10		22		2	A3*B1*	
MS	0146	92	2	-	22	S	1*	A3*B1*	5
MV	4326		3	2	27	S	1*	A3*B1*	4
FS	3493	253	2	2	27	S		A3*B1*	7
FS	1825	2	2	2	27	S	1*	A3*B1*	2
WC	2102		2		27	F	1*	A3*B1*	
WC	3585		10		27	S	1*	A3*B1*	
WP	0089	5	3		27	S	1*	A3*B1*	
WP	0187	14	3	2	27	S		A3*B1*	
WP	0436	32	2		27			A3*B1*	
WP	0402	35	2	3	27	S	1*	A3*B1*	
FS	2650	51	2	-	35	S	27	A3*B1*	
FS	1636	29	2	4	35	S	1*	A3*B1*	
FS	3447	254	32	4	35	S		A3*B1*	
WC	2036		3		35	S	1*	A3*B1*	
WC	3533		11		35	S	1*	A3*B1*	
WP	0134	8	3		35	S	1*	A3*B1*	
WP	0153	10	11		35	S	1*	A3*B1*	
WP	0154	11	31		35	S	1*	A3*B1*	
WP	0408	28	31		35			A3*B1*	
WP	0402	35	2	-	35	S	1*	A3*B1*	
MS	0513	95	28	4	35	F	1*	A3*B1*	2
FS	3484	254	1	6	37	S		A3*B1*	
WC	6305		3		37	S	1*	A3*B1*	
WC	3203		3		37	S	1*	A3*B1*	
MS	0302	50	1	-	37	S	1*	A3*B1*	
FS	2647	62	2	-	38	S	1*	A3*B1*	
MV	4239		31		39	S	1*	A3*B1*	5
MV	4239		31		39	S	1*	A3*B1*	5
MV	4312		24	2	40	S	1*	A3*B1*	5
MV	4326		25	3	40	S	1*	A3*B1*	4
FS	2620	51	2	2	40	S	1	A3*B1*	
FS	1760	52	3	-	40			A3*B1*	5
FS	2935	90	24	7	40	S	1*	A3*B1*	
FS	2933	90	30	7	40	S	1*	A3*B1*	
FS	3401	252	25	3	40	S	1*	A3*B1*	
FS	3236	238	2	3	40	S	1*	A3*B1*	
WC	3533		1		40	S	1*	A3*B1*	
WC	3547		1		40	S	1*	A3*B1*	
WC	3402		32		40	S	1*	A3*B1*	
WP	0017	2	24		40		1*	A3*B1*	
WP	0153	10	24		40	S	1*	A3*B1*	
WP	0154	11	2		40	F	1*	A3*B1*	
WP	0311	19	1		40	S	1*	A3*B1*	
WP	0366	23	32		40	S		A3*B1*	
MS	0321	43	2	-	40	S	1*	A3*B1*	27
MS	0415	77	28	3	40	S	1*	A3*B1*	4
MS	0499	77	28	3	40	S	1*	A3*B1*	4
MS	0514	95	28	-	40	S	1*	A3*B1*	7
MV	4238		23		41	F	1*	A3*B1*	6
WP	0461	33	3		44		1*	A3*B1*	
WP	0475	34	3	-	44	F	1*	A3*B1*	
MS	0054	106	29	7	44		1*	A3*B1*	7
MS	0087		2	-	44	F	1*	A3*B1*	
MS	0090		1	-	44	F	1*	A3*B1*	

MS	0302	50	29	-	44	S	1*	A3*B1*				
WP	0312	19	2		49	S	1*	A3*B1*				
FS	2477	234	3	-	7	S		A3*B2				
BA	1432	207	2	3	15	S		A3*B2				
MS	0542	123	29	-	39	S	2	A3*B2	7			
FS	2478	234	2	3	40	S		A3*B2				
FS	2270	240	2	3	40	S	1*	A3*B2				
MS	0293	23	7	7	40	S	1	A3*B2	7			
MS	0153	63	11	4	44	S	1*	A3*B2	4			
MS	0270	60	28	-	44	S	1*	A3*B2	3			
MS	0311	50	2	-	45	S	1*	A3*B2	2			
MS	0178	12	26	1	51	S	1*	A3*B2	1			
WC	5275		1		7	S	1*	A3*B2 B1*				
FS	2287	225	2	-	5	S		A3*B3~				
BA	1268	104	2	3	15			A3*B3~				
FS	2346	231	31	-	15		1*	A3*B3~				
FS	3404	252	2	-	15	S	1*	A3*B3~				
FS	3103	248	2	3	15	S	1*	A3*B3~				
FS	3267	241	2	3	15	S	1*	A3*B3~				
WC	1218		2		15	S	1*	A3*B3~			+	
WC	1103		2		15	S	1*	A3*B3~			+	
WP	0392	27	3		37	F	1*	A3*B3~				
WC	3664		2		40	S	1*	A3*B3~	-			
WC	1351		2		64	S	1*	A3*B3~		+		
FS	1955	57	29	-	12	S	2	A3* B1*	7			
MS	0579	103	28	-	-	S	2	A4 B2	4			
FS	1690	19	28	-	5	S	1*	A4 B2				
WC	3900		2		5	S	2	A4 B2				
WC	3590		31		5	S	1*	A4 B2				
MV	4306		1	4	7	S	1*	A4 B2	1	+	+	
FS	2649	62	2	-	7	S	2	A4 B2				
WC	7319		1		7	S	2	A4 B2				
WP	0148	10	3		7	S	1*	A4 B2				
MS	0267	52	2	-	7	S	1*	A4 B2	-			
MS	0290	28	3	-	7			A4 B2	7			
MS	0478	90	2	3	7	S	1*	A4 B2	4			
WC	5063		1		8	S	27	A4 B2				
FS	3076	248	28		x	S	27	A4 B2				
LA	0146		2	37	12	S		A4 B2				
FS	1996	42	1	5	12	S	2	A4 B2				
WC	3270		2		12	S	2	A4 B2				
WC	3269		2		12	S	2	A4 B2				
WP	0002	1	23		12	S	1*	A4 B2				
WP	0036	4	2	-	12	S	2	A4 B2				
MV	4291		2	3	15	S	2	A4 B2	4			
FS	2225	35	24	3	15	S		A4 B2				
FS	3385	251	1	3	15	S	2	A4 B2	4			
WC	3803		9		15	S	2	A4 B2				
WP	0305	20	2	3	15	S	2	A4 B2	4			
MS	0212	32	31	3	15	S	2	A4 B2	2			
MS	0274	27	24	3	15	S	1	A4 B2	2			
MS	0292	23	2	3	15	S	2	A4 B2	4			
MS	0441	61	1	-	15	S	2	A4 B2	47			
MS	0567	17	26	-	16	S	1*	A4 B2	4			
FS	2373	218	10	-	17	S	2	A4 B2				
FS	3402	253	24	-	18	S		A4 B2	7			
FS	3164	248	25	-	18	S	27	A4 B2				
WC	2001		10		18	S	1*	A4 B2				
WC	3204		10		18	S	1*	A4 B2				
WC	1235		25		18	S	1*	A4 B2				

WP	0308	20	26		18	S	0	A4	B2	2		
WP	0418	30	25		18	S		A4	B2			
MS	0318	36	32	-	18	S	1*	A4	B2	2		
MS	0330	61	25	-	18	S	1*	A4	B2	2		
MS	0362	36	25	-	18	S	1*	A4	B2	2		
MS	0397	70	25	-	18	S	1*	A4	B2			
MS	0477	90	25	-	18	S	1*	A4	B2	2		
WC	3115		3		22	S	2	A4	B2			
MS	0212	32	1	7	22	S	2	A4	B2		+	
MS	0562	49	11	3	22	S	1*	A4	B2	4		
FS	2257	36	28	2	27	S	1*	A4	B2	2		
MS	0265	17	2	1	27	S	1*	A4	B2	7	+	
MS	0271	60	11	1	27	S	1*	A4	B2	-		
MS	0322	43	2	2	27	S	1*	A4	B2	-		
MS	0364	36	25	2	27	S	1*	A4	B2	4		
MS	0376	29	3	1	27	S	1*	A4	B2			
MS	0525	85	2	-	27	S	1*	A4	B2	4		
FS	3371	251	3	4	35	S	2	A4	B2	4		
WC	3191		11		35	S	1*	A4	B2			
WP	0092	5	11		35	S	1*	A4	B2			
MS	0299	34	24	4	35	S	1*	A4	B2	1		
MS	0327	68	11	4	35	S	2	A4	B2	4		
MS	0437	99	24	4	35	S	1*	A4	B2	7		
MV	4295		1	3	37	S	1*	A4	B2	5	+	
FS	2020	54	3	7	39	S	1*	A4	B2			
FS	3414	252	2	3	40	S	2	A4	B2			
BB	1213	241	1	-	40	S	1*	A4	B2			
WP	0001	1	2		40	S	1*	A4	B2			
WP	0180	13	2		40	S		A4	B2			
WP	0255	17	9		40	S	1*	A4	B2			
MS	0107	18	2	2	40	S	2	A4	B2	-		
MS	0209	55	2	3	40	S	1*	A4	B2	4		
MS	0354	49	24	-	44	F	1*	A4	B2			
MS	0072	106	1		52	S	1*	A4	B2			
MS	0469	90	29	-	52	S	1*	A4	B2	4		
FS	3535	257	11		62	S		A4	B2			
MS	0143	47	11	3	62	S	1*	A4	B2	2		
MS	0537	87	24	3	62	S	1*	A4	B2	-		
MV	4313		3		7	S	1*	A4	B4~	5		
WC	1101		11		7	S	2	A4	B4~		0	
MV	4209		2	3	22	S		A4	B4~	4		
FS	2407	38	2	3	22	S		A4	B4~		+	
FS	2136	23	11	3	22	S	2	A4	B4~			
FS	3504	249	2	3	22	S	2	A4	B4~			
WP	0137	8	1		22	S	2	A4	B4~			
WP	0308	20	3		22	S	2	A4	B4~	x		
WP	0345	22	2		22	S	2	A4	B4~			
WP	0347	22	1		22	S	2	A4	B4~			
MS	0177	12	26	3	22	S	2	A4	B4~	5		
MS	0195	17	24	3	22	S	2	A4	B4~	3		
MS	0395	73	2	3	22	S	2	A4	B4~	-		
MS	0509	34	2	3	40	S		A4	B4~	7		
MS	0010	58	11	3	55	S	2	A4	B4~	-		
MS	0537	87	2	-	27	S	1	A4*	B2*	-		
WP	0251	17	2		15	S	1*	A6	B1			
FS	1666	22	11	-	5	S	1*	A6	B1			
FS	2632	58	2	-	7	S	1*	A6	B1			
FS	3155	243	28	-	12	S	1*	A6	B1			
MV	4270		2	6	17	S	1*	A6	B1	7		
BA	1100	56	1		17	S	1*	A6	B1	7		

FS	2633	58	2	-	17	S	1*	A6 B1			
FS	1693	220	1	6	17		1*	A6 B1	7		
FS	2288	225	3	4	17	S		A6 B1			
FS	3491	253	29	6	17	S		A6 B1	7		
FS	3571	258	2	-	17	S	1*	A6 B1	7		
FS	2784	247	1	6	17	S	1	A6 B1			
FS	3060	247	2	-	17	S	1*	A6 B1			
FS	2911	247	1	-	17	S	1	A6 B1			
FS	2064	86	1	2	17	S	1*	A6 B1			
WP	0388	26	1		17	S		A6 B1			
MS	0002	116	2	2	17	S	1*	A6 B1	7		
MS	0144	41	1	-	17	S	1*	A6 B1	4		
MS	0182	13	1	-	17	S	1*	A6 B1	7		
MS	0437	99	1	-	17	S	1*	A6 B1	7		
MS	0452	88	2	-	17	S	1*	A6 B1	7		
MS	0546	121	1	-	17	S	1*	A6 B1	7		
MS	0301	96	28	2	27	S	1*	A6 B1	7		
FS	1765	21	3	4	35	S	1*	A6 B1			
MS	0158	45	1	-	37	F	1*	A6 B1		+	
MS	0136	92	11	-	7	S	1*	AQOB1	2		
MS	0533	87	3	-	7	S	1*	AQOB1	3	0	
MS	0595	19	3	-	7			AQOB1	3	0	
MV	4262		2		8	S	1*	AQOB1	3		
FS	2297	57	1	-	8	S	1	AQOB1	3		
MV	4271		1		8	S	1*	AQOB1	3		
MV	4296		1		8	S	1*	AQOB1	3	0	
MV	4296		1		8	S	1*	AQOB1	3	0	
LA	0125		1		8	S	1*	AQOB1		0	
LA	0152		1		8	S	1*	AQOB1	3		
LA	0168		1		8	S		AQOB1			
LA	0177		1		8	S		AQOB1			
LA	0195		1		8	S	1*	AQOB1			
BA	1100	56	1		8	S	1*	AQOB1	3		
FS	1655	34	1	-	8	S		AQOB1	3		
FS	1655	34	1	-	8	S		AQOB1	7		
FS	1109	25	1	3	8	S	1*	AQOB1			
FS	1667	22	1	-	8	S	1*	AQOB1			
FS	2854	66	1	-	8	S	1*	AQOB1			
BC	1187	91	1		8	S		AQOB1	3		
FS	2974	80	1	-	8	S		AQOB1			
FS	2974	80	1	-	8	S		AQOB1			
BC	1189	91	1		8	S		AQOB1	3		
BC	1187	91	1		8	S		AQOB1	6		
BA	1435	208	1	-	8	S	1*	AQOB1			
FS	2040	219	1	?	8	S	1*	AQOB1			
FS	2320	232	2	-	8	S	1*	AQOB1			
FS	2374	218	1	-	8	S	1	AQOB1			
FS	1692	19	1	-	8	S	1*	AQOB1			
FS	3112	249	1	-	8	S	1*	AQOB1			
FS	3112	249	3	-	8	S	1*	AQOB1			
FS	3160	243	1	-	8	S	1*	AQOB1			
FS	3119	248	1		8	S		AQOB1			
FS	2792	63	2	-	8	S	1*	AQOB1			
FS	2792	63	2	-	8	S	1*	AQOB1			
WC	3318		1		8	S	1*	AQOB1		0	
WC	3153		1		8	S	1*	AQOB1		0	
WC	3095		1		8	S	1*	AQOB1		0	
WC	1064		1		8	S	1*	AQOB1		0	
WC	3043		1		8	S	1*	AQOB1			
WC	1131		1		8	S	1*	AQOB1		0	

WC	3057		1		8	S	1*	AQOB1		0	
WC	3159		1		8	S	1	AQOB1		0	
WC	3148		1		8	S	1*	AQOB1		0	
WC	3308		1		8	S	1*	AQOB1			
WC	3090		1		8	S	1	AQOB1			
WC	3269		1		8	S	1	AQOB1			
WC	1064		2		8	S	1	AQOB1		0	
WC	3288		3		8	S	1	AQOB1		0	
WC	3708		3		8	S	1	AQOB1		0	
WC	3708		3		8	S	1	AQOB1		0	
WP	0010	2	1		8	S	1*	AQOB1			
WP	0015	2	10		8	F		AQOB1			
WP	0028	3	1		8	S	1*	AQOB1			
WP	0147	10	1		8	S		AQOB1			
WP	0219	16	1		8	S	1*	AQOB1			
WP	0251	17	10		8	S	1*	AQOB1			
WP	0262	17	10		8	S	1*	AQOB1			
WP	0380	25	1	-	8	S	1*	AQOB1			
MS	0001	116	1	-	8	S	1*	AQOB1	3		
MS	0057	75	1	-	8	S	1*	AQOB1	1		
MS	0069	58	3	47	8	S	1*	AQOB1	7	0	
MS	0107	18	1	-	8	S	1	AQOB1	3		
MS	0115	65	2	-	8	S	1*	AQOB1	3	0	
MS	0116	65	1	-	8	S	1*	AQOB1	3	0	
MS	0142	47	24	-	8	S	1*	AQOB1			
MS	0159	57	1	-	8	S	1*	AQOB1	37	0	
MS	0159	57	1	-	8	S	1*	AQOB1	3	-	
MS	0165	11	1	-	8	S	1*	AQOB1	3		
MS	0168	10	1	-	8	S	1*	AQOB1	3		
MS	0173	13	1	-	8	S	1*	AQOB1	3		
MS	0193	16	1	-	8	S	1*	AQOB1	3		
MS	0200	34	1	-	8	S	1*	AQOB1	3		
MS	0201	106	1	3	8	S	1*	AQOB1	3	0	
MS	0204	23	1	-	8	S	1*	AQOB1	3		
MS	0229	21	1	-	8	S	1*	AQOB1	3		
MS	0259	52	1	-	8	S	1*	AQOB1	3		
MS	0261	55	1	-	8	S	1*	AQOB1	3		
MS	0265	17	1	-	8	S	1	AQOB1	3	0	
MS	0273	27	1	-	8	S	1	AQOB1	3		
MS	0352	47	1	-	8	S	1*	AQOB1	3		
MS	0377	37	1	-	8	S	1*	AQOB1	3		
MS	0392	70	1	-	8	S	1*	AQOB1	2		
MS	0428	85	1	-	8	S	1*	AQOB1	3		
MS	0429	99	1	-	8	S	1*	AQOB1	-		
MS	0441	61	2	-	8	S	1	AQOB1	3		
MS	0469	90	1	-	8	S	1*	AQOB1	3		
MS	0495	99	34	-	8	S	1*	AQOB1	3		
MS	0500	119	1	-	8	S	1*	AQOB1	3		
MS	0516	89	1	-	8	S	1*	AQOB1	7		
MS	0529	85	1	-	8	S	1*	AQOB1	3		
MS	0533	87	1	-	8	S	1*	AQOB1	3	0	
MS	0561	123	1	-	8	S	1*	AQOB1	3		
MS	0581	103	3	-	8	S	1*	AQOB1	3		
MS	0596	19	1	-	8			AQOB1	3	0	
MS	0264	17	17	7	87	S	1	AQOB1	37	0	
WC	3480		10		12	F	1*	AQOB1			
WC	3497		33		12	S	1*	AQOB1		0	
MS	0425	75	30	-	13	S	1*	AQOB1	7		
LA	0125		9	6	14	S	1*	AQOB1		0	
WC	3388		11		14	S	1*	AQOB1			

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WC   3498          9      15  S  1    AQOB1        0
WC   2089          3      16  S  1*   AQOB1        0
MS   0231     47   9   -  16  S  1*   AQOB1      7
FS   2003    219   1   -  17  S  1*   AQOB1
MS   0069     58   1      17  S  1*   AQOB1      7 0
MS   0211     32   2   -  17  S  1    AQOB1      4 0
WC   3416         30      18  S  1    AQOB1        0
BC   1189     91   2      21  S       AQOB1      4
WP   0419     30   2      21  S1      AQOB1
FS   3183    243   3   4  35  S  1*   AQOB1
FS   3139    243   3   4  35  S  1*   AQOB1
FS   3080    248   2      38  S       AQOB1
WP   0096      5   2      40  S  1*   AQOB1
MV   4305          2      41  F  1*   AQOB1      7  *  +
MS   0574    103   2   2  44  F  1*   AQOB1      1  0
MS   0195     17  29   -  49  S  1    AQOB1        7
MS   0211     32   2   -  49  S  1*   AQOB1      4  0
WC   5320          9       5  S  1    AQOB2        0
FS   1051     25   2   1  40  S  1*   AQOB2
WP   0100      6   2      40  S       AQOB2
WP   0458     33   2   3  40  S  1*   AQOB2
WP   0458     33  11   -  40  S  1*   AQOB2
MS   0201    106  28   7  40  S  1*   AQOB2      1  0
LA   0110          9   2  41  S  1*   AQOB2      4  0
MS   0327     68   3   3  60  S  1    AQOB2      -
MS   0574    103   1   3  60  S  1*   AQOB2      4  0
WP   0219     16   3      40  F  1*   AQOB2
WC   3834          2      12  F  1* A2*A3 BQO        P  0
WC   1221          2      12  F  1* A2*A3 BQO           0
WC   1011          2      12  S  1* A2*A3 BQO           0
WP   0419     30   2      13  S     A2*A3 BQO
WC   1042          2      44  F  1  A2*A3 BQO           0
WP   0242     16   2      35  F  1* A3 A2 BQO
WP   0245     16   2      35  F  1* A3 A2 BQO
WC   1046          3      39  F  1  A3 A2 BQO        P  0
WP   0013      2   2       7     1* A3*A2 B1*
WP   0082      5   2      15  F     A3*A2 B1*
FS   2966    247   3   4  35  F  1* A3*A2 D1*
WP   0371     24   3      35     1* A3*A2 B1*
WP   0400     28  32      35        A3*A2 B1*
MS   0033    113   2   4  35  F  1* A3*A2 B1*      1
MS   0443     92   3   4  35  F  1* A3*A2 B1*      1
MS   0492     96   1   4  35  F  1* A3*A2 B2*      1
WC   3322         31       5  F  1* A3*A2 BQO
FS   2407     38   2   -  12  S     A3*A2 BQO
MS   0162     12   3   4  35  F  1  A3*A2 BQO      1
MS   0177     12   3   4  35  F  1  A3*A2 BQO      1
MS   0178     12   2   4  35  F  1  A3*A2 BQO      1
MS   0208     55   3   4  35  F  1* A3*A2 BQO      5
MS   0369     68  11   4  35  F  1  A3*A2 BQO      4
MS   0555    116   3   4  35  F  1* A3*A2 BQO      1
MS   0412    119  24   -  39  F  1* A3*A2 BQO      1
FS   3533    257  30/     51  F     A3*A2 BQO

DTR> EXIT

Ready
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