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# The origin of the very variable haemolytic activities of the common human complement component C4 allotypes including C4-A6

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**The human complement component C4 occurs in many different forms which show big differences in their haemolytic activities. This phenomenon seems likely to be of considerable importance both physiologically and pathologically. C4 is coded by duplicated genes between HLA-D and HLA-B loci in the major histocompatibility complex in man. Several fold differences in haemolytic activity between products of the two loci C4-A and C4-B have been correlated with changes of six amino acid residues in this large protein of 1722 residues and with differences of several fold in the covalent binding of C4 to antibody-antigen aggregates. Some allotypes of one locus also differ markedly, notably C4-A6 which has 1/10th the haemolytic activity of other C4-A allotypes. A monoclonal antibody affinity column has been prepared which is able to separate C4-A from C4-B proteins and, using serum from an individual expressing only the C4-A6 allele at the C4-A locus, C4-A6 protein has been prepared. Investigation has shown C4-A6 to have the same reactivity as other C4-A allotypes except in the formation of the complex protease, C5 convertase. This protease is formed from C4, C2 and C3 and if C4-A6 is used it has ~1/5th the catalytic activity compared with other C4-A allotype. Allelic differences in sequence identified in C4 proteins so far are few and it is probable that the big difference in catalytic activity of C5 convertase is caused by very small changes in structure.**

*Key words:* allotypes/C4/complement/haemolytic activity

## Introduction

Genes coding for three components of complement C2, C4 and factor B are in the class III region between HLA-D (class II) and HLA-B (class I) in the major histocompatibility complex in man. These loci are exceptionally polymorphic and this is true for C4 which has a duplicated locus (C4-A and C4-B) with many alleles at each. Thirty-five alleles have been distinguished by charge and antigenic specificity (Mauff *et al.*, 1983) but serological studies (Giles, 1985a, 1985b; Giles *et al.*, 1985), restriction enzyme digestion (Whitehead *et al.*, 1984) and nucleotide sequencing of the genes (Belt *et al.*, 1985) are subdividing the allelic forms recognised so far and the total number of alleles is likely to be much higher. There are big differences in activity between C4-A and C4-B allotypes. As many individuals are heterozygous at one or both loci, the range of reactivity of several forms of C4 may be of biological advantage in the rapid destruction and removal of pathogens with widely different surface structures. In certain circumstances, it may also offer increased risk of autoimmune damage (Porter, 1983) and there is a correlation between susceptibility to disease of this type and particular associations of alleles in the HLA-B to HLA-D region (reviewed by O'Neill, 1984). There is further interest in the variability of C4 as the structural differences between the different allotypes is very small, with all accounted for by changes in perhaps <1% of the total amino acid sequence of 1722 residues (Belt *et al.*, 1984, 1985). Correlation of sequence and reactivity of C4 should provide a direct guide to the structural basis of the different biological activities.

The haemolytic activity of C4 depends upon series of steps of activation, interaction and inactivation in which C4 interacts with eight different proteins (reviewed in Reid and Porter, 1981; Porter, 1984). C4-B allotypes are several fold more active in haemolytic assay than C4-A allotypes and this was shown to be due to a difference in the covalent binding of activated $\bar{C4}$ to antibody-coated red cells (Isenman and Young, 1984; Law *et al.*, 1984a). This occurs through a reactive acyl group released from an intrachain thioester bond in the $\alpha$-chain when C4 is activated by splitting of a peptide bond near the N terminus of the $\alpha$-chain. It was shown further that C4-B formed a covalent bond much more rapidly than C4-A with hydroxyl groups in glycerol and that the reverse was true for reaction with amino groups in glycine (Isenman and Young, 1984; Law *et al.*, 1984a). This agreed with the greater reaction of C4-B with the polysaccharide-covered red cells and of C4-A with aggregates formed with the polysaccharide-free protein, serum albumin.

There are also differences in haemolytic activity between allotypes of the same locus, notably C4-A6 which has been reported to have little or no activity in the overlay assay (Teisberg *et al.*, 1980; O'Neill *et al.*, 1980). It has now been possible to investigate the origin of this difference because a monoclonal affinity column has been prepared which can separate C4-A from C4-B allotypes. Using serum from an individual expressing only C4-A6 at the C4-A locus, the properties of this protein have been studied. It proved to be identical to other C4-A allotypes in all its interactions except in the formation of the complex protease C5 convertase. This protease is formed from C2 and C3 complexed with C4 which is covalently bound to the activating aggregates. If formed with C4-A6, the convertase splits C5 at only ~1/5th the rate than if formed from other allotypes of C4. As the formation of the lytic complex from the terminal components of the complement cascade depends on the rate of activation of C5, this finding explains the low haemolytic activity of C4-A6.

## Results

### *Separation of C4-A and C4-B allelic proteins*

When 10 ml of serum containing both C4-A and C4-B were loaded on to an affinity column of monoclonal anti-C4 antibody (L003) coupled to Sepharose, the column washed and bound protein eluted by a buffered pH gradient as described in Materials and methods, two protein peaks were observed (Figure 1). The fractions were analysed on SDS-PAGE by the technique of Roos *et al.* (1982) in which the $\alpha$-chain of C4-A has a slightly slower mobility than that of C4-B (Figure 1). This showed the first peak to contain C4-A and the second C4-B. C. Giles typed the two

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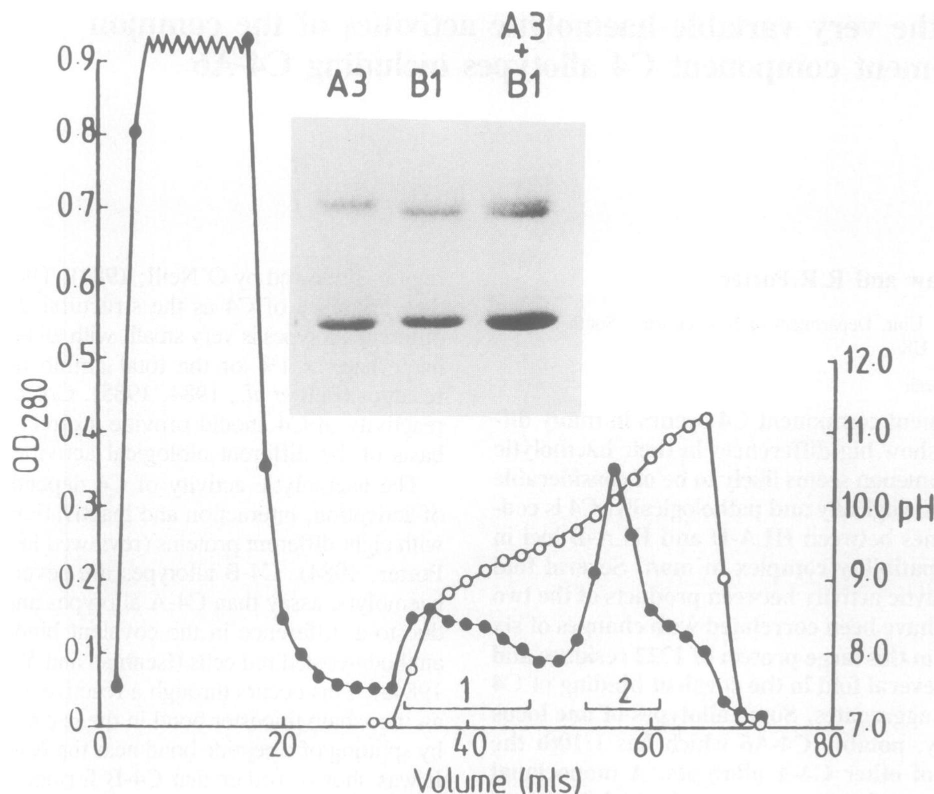


**Fig. 1.** Purification of C4 allotypes. Elution profile of C4-A3 and C4-B1 (●, $OD_{280}$ values) from donor RP from the L003 monoclonal antibody column with a pH gradient (○). Pool 1 contained C4-A3 and pool 2 contained C4-B1. Inset are SDS-PAGE gels of the purified proteins after concentration on DEAE Sephacel, only the α- and β-chains are shown (Materials and methods).

peaks using anti-Rodgers and anti-Chido serum specific for C4-A and C4-B, respectively. There was slight contamination detected of each by the other using this criteria but none could be seen on SDS-PAGE. If serum from individuals expressing only one allele at either the C4-A or C4-B locus were fractionated by this method, single allelic proteins could be obtained of sufficient purity to investigate their biological and chemical activities. The total yield of C4 protein was not less than 80%, 1 – 3 mg/10 ml serum depending on the initial concentration in the blood. Using this method, the following C4 allelic proteins were obtained: C4-A3, A4, A6 and C4-B1, B2 from several donors.

*Comparison of the properties of the C4-A6 protein with those of other C4-A and C4-B allotypes*

*Haemolytic assays.* The C4-A6 protein has been reported to have little or no haemolytic activity when tested using a haemolytic overlay assay after electrophoresis in agarose gel (Teisberg *et al.*, 1980; O'Neill *et al.*, 1980). When the C4 allotypes were assayed for haemolytic activity by three different forms of *in vitro* assay, using either C4 deficient guinea-pig serum, human serum depleted of C4 or EAC1 cells to which C4, then C2 and the late components of complement were added, the relative titres agreed with each other and with the haemolytic overlay assay (Table I). The absolute values varied 10-fold but in each assay C4-B1 and C4-B2 had several times higher activity than C4-A3 and C4-A4 and 50 – 200 times higher than C4-A6. This establishes that the very low haemolytic activity of the C4-A6 allotype is genuine and is not in any way an artefact of the type of assay used.

*Binding of C4 allelic proteins to antibody-coated red cells and small molecules.* In view of the previous finding that the difference between the haemolytic activities of C4-A and C4-B proteins correlated with the efficiency of covalent binding of C4 to EAC1 cells (Law *et al.*, 1984a; Isenman and Young, 1984) the binding of C4-A6 to EAC1 cells was compared with that of the other allotypes. As found previously, the binding of the C4-A allotypes was less than half that of the C4-B allotypes, but there was little difference between the binding of C4-A6 and C4-A3 (Figure 2).

The membrane polypeptides of EAC14 cells made with $^{125}$I-labelled C4-A3, A6 and B1 were analysed by SDS-PAGE followed by autoradiography (Figure 3). The radioactive bands above the α′-chain of C4 represented the covalent complexes between the C4α′-chain and other cell-surface proteins. The membrane polypeptide patterns between EAC14 cells made with C4-B1 and those made with C4-A3 and C4-A6 were different, indicating

**Table I.** Specific haemolytic activity of different C4 allotypes

| Donor | C4 allotype | | Protein isolated | C4 titre (units/μg) | | |
|---|---|---|---|---|---|---|
| | C4-A | C4-B | | GP-C4Def[a] | Human-C4Def[b] | Classical assay[c] |
| RP | 3,3 | 1,1 | A3 | 395 | 31 | 217 |
| | | | B1 | 1057 | 70 | 1570 |
| HP | 6,Q0 | 1,1 | A6 | 43 | 2.8 | 7.8 |
| | | | B1 | 1011 | 123 | 1770 |
| CA | 4,4 | 2,Q0 | A4 | 348 | 31 | 200 |
| | | | B2 | 1023 | 82 | 1660 |

[a]C4-deficient guinea-pig serum from guinea-pig genetically deficient in C4 used for assay.
[b]C4-deficient human serum by immunoabsorption of human serum with Sepharose-bound anti-C4 antibody used for assay.
[c]C4 was assayed by its addition to EAC1 cells followed by C2 and C-EDTA by stepwise addition (Gigli *et al.*, 1977).

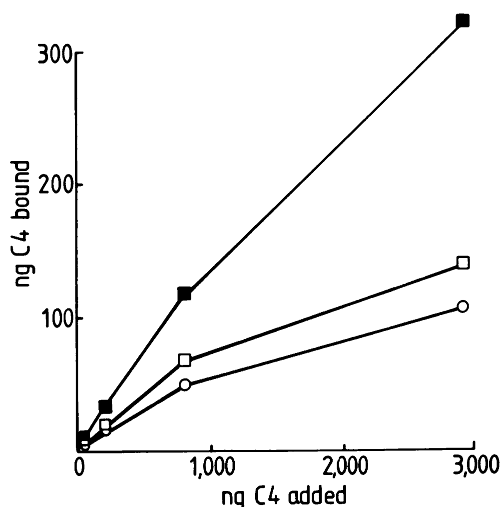


**Fig. 2.** C4 binding to EAC1 cells. $^{125}$I-labelled C4-A3 (□); C4-A6 (○); and C4-B1 (■) were incubated with EAC1 cells at 37°C for 15 min. After washing, cells were counted and the bound C4 calculated. Background binding to EA cells was <10% of specific binding.

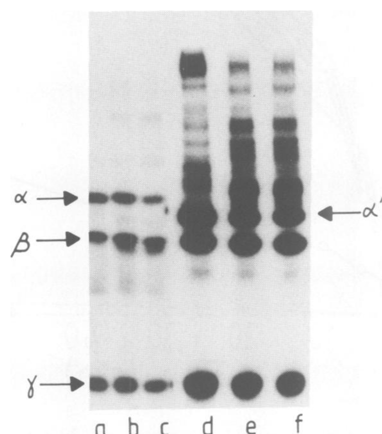


**Fig. 3.** Autoradiograph of [$^{125}$I]C4 bound to red cell membrane. EAC14 cells were prepared carrying ~3000 molecules per cell of [$^{125}$I]C4-B1, C4-A3 and C4-A6. Cell membranes were reduced and run on SDS-PAGE and the gel autoradiographed. Shown on **tracks a–c** are the C4 proteins B1, A3, and A6; and **tracks d–f** are the membrane polypeptides with covalently bound [$^{125}$I]C4 from EAC14 cells prepared from C4-B1, A3 and A6, respectively.

the B allotype of C4b binds covalently to a different set of membrane proteins when compared with the A allotypes. The C4-A6 and C4-A3 allotypes bind to a very similar, if not identical, set of proteins. The low haemolytic activity of C4-A6 is therefore probably not due to its selective reactivity with membrane macromolecules.

There are very large differences in the reaction rates of C4-A3 and C4-B1, activated by C1s, when binding to the amino group of glycine (300-fold) or the hydroxyl group of glycerol (10-fold) (Table II). However, when the relative rates of the different A allotypes were compared and also those of the different B allotypes the differences were small (Table III). Differences such as that between the two C4-B1 allotypes (1:1.58) are unlikely to be significant. However, the two proteins typed B1 from different individuals may not be identical in amino acid sequence (Belt *et al.*, 1985) and the small differences found may be valid. It is clear, however, that the low haemolytic activity of C4-A6 is not due to low efficiency of the covalent binding activity which

**Table II.** The reaction rates of [$^3$H]glycine and [$^3$H]glycerol with C4-A3 and C4-B1

| | $k'/k_o$ ($M^{-1}$) | |
|---|---|---|
| | Glycine | Glycerol |
| C4-A3 | 12 300 | 1.27 |
| C4-B1 | 39.6 | 11.2 |

The binding reaction was carried out in 10 mM sodium phosphate, 125 mM NaCl, 0.5 mM EDTA, pH 7.5. Binding efficiency, defined as the fraction of C4 bound with radioactive small molecules, was determined at different concentrations of glycine and glycerol. The concentration of glycine was 40 μM and 2.5 mM, and that of glycerol was 20 mM and 10 mM for C4-A3 and C4-B1, respectively. Binding efficiency (BE) is related to the reaction rate by the equation: $BE = k'[G]/(k_o + k'[G])$ (Law *et al.*, 1984b); where $k_o$ is the first-order reaction rate of the covalent binding site of C4 to water, $k'$ the second-order reaction rate with substrate molecule; and [G] the concentration of the substrate molecule under study, in this case glycine or glycerol. The $k'/k_o$ values for the reaction of glycine and glycerol with C4-A3 and C4-B1 were calculated from the measured binding efficiency accordingly.

**Table III.**

| | Glycine | Glycerol |
|---|---|---|
| (A) The relative reaction rates of C4-A4 and C4-A6 to glycine and glycerol with respect to C4-A3[a] | | |
| C4-A3 | 1[c] | 1[c] |
| C4-A4 | 1.20 ± 0.43 | 0.99 ± 0.47 |
| C4-A6 | 1.19 ± 0.44 | 0.95 ± 0.13 |
| (B) The relative reaction rates of C4-B1-HP and C4-B2 to glycine and glycerol with respect to C4-B1-RP[b] | | |
| C4-B1-RP | 1[c] | 1[c] |
| C4-B1-HP | 1.58 ± 0.31 | 1.01 ± 0.09 |
| C4-B2 | 0.82 ± 0.05 | 0.98 ± 0.08 |

[a]The ratios of $(k'/k_o)_{C4\text{-}A4} : (k'/k_o)_{C4\text{-}A3}$ and $(k'/k_o)_{C4\text{-}A6} : (k'/k_o)_{C4\text{-}A3}$ for glycine and glycerol were determined. Shown are the values averaged over several measurements.
[b]Values of $(k'/k_o)_{C4\text{-}B1\text{-}HP} : (k'/k_o)_{C4\text{-}B1\text{-}RP}$ and $(k'/k_o)_{C4\text{-}B2} : (k'/k_o)_{C4\text{-}B1\text{-}RP}$ : for glycine and glycerol are shown.
[c]Standards: values = 1 by definition.

is very close to that of the other C4-A allotypes.

When other interactions of the C4 allotypes were compared, activation by C1s which releases a peptide from the N terminus of the α-chain was the same for all, as was inactivation by factor I and the C4 binding protein which hydrolyses two bonds in the α'-chain (Table IV).

*Formation of C3 and C5 convertases with C4 allotypes*

C3 convertases were formed using EAC1 cells and different C4 allotypes as described in Materials and methods. Conditions were such that, in all cases, ~3000 molecules of C4 were bound per cell. Human C2 was added together with human C3 and the rate of C3 hydrolysis estimated after SDS-PAGE. Under these conditions no significant difference in C3 convertase activity was observed (Figure 4a).

For a similar assay of C5 convertase $^{125}$I-labelled C5 was used, as the low level of protein used could not be distinguished from C3 and other proteins on electrophoresis. Autoradiography and scanning gave the rate of hydrolysis of the C5 (Figure 4b). This showed a marked difference in the C5 convertase activities in which with C4-A6 the initial rate of C5 hydrolysis was only ~1/5th that of the other C4-A and C4-B allotypes. As lysis of the red cells depends upon the rate of formation of the lytic com-

**Table IV.**

(A) Hydrolysis of C4 by C$\bar{1}$s

| C$\bar{1}$s (ng) | % cleavage of C4 α-chain[a] | | |
|---|---|---|---|
| | C4-B1-RP | C4-A3-RP | C4-A6-HP |
| 100 | 100 | 100 | 100 |
| 10 | 84 | 88 | 83 |
| 1 | 30 | 33 | 36 |
| 0.1 | 13 | 8 | 9 |

(B) Hydrolysis of C4b by factor I with C4 binding protein

| Factor I (ng) | % cleavage of C4 α′-chain[b] | | |
|---|---|---|---|
| 1000 | 78 | 78 | 76 |
| 100 | 44 | 44 | 42 |
| 10 | 14 | 15 | 12 |

[a]12 μg of C4 was incubated with C$\bar{1}$s at 37°C for 15 min in 25 μl phosphate-buffered saline. Samples were reduced and run on SDS-PAGE. The degree of hydrolysis of the C4 α-chain was determined by gel scanning.
[b]12 μg of C4b was incubated with factor I and 5 μg of C4 binding protein at 37°C for 15 min in phosphate-buffered saline. Samples were analysed by SDS-PAGE and the degree of cleavage of the C4 α′-chain determined by gel scanning.

plex and this is initiated by activation of C5, this difference in activity explains adequately the low haemolytic activity of the C4-A6 allotype.

## Discussion

The monoclonal antibody L003 shows a different affinity for C4-A allotypes, which carry the Rodgers antigenic determinant, from C4-B allotypes, which have the Chido determinant. This made possible the preparation of an affinity column able to separate C4-A from C4-B proteins probably in >90% purity, sufficient to study their properties. As further subdivision of the antigenic properties of both the C4-A and C4-B allotypes are being found (Giles, 1985a, 1985b) it is unlikely to be able to separate all the antigenic variants which occur. However, it is effective in isolating individual allotypes from the serum of donors expressing only a single C4-A or C4-B protein.

There may be as many as 50 different allelic forms of human C4 coded in two loci, comparable with the polymorphism observed in the adjacent Class I and Class II loci in HLA. A special feature of C4, however, is that the polymorphism appears to arise from the changes of very few nucleotides, probably <1% of the 5223 nucleotide long coding region. Most of the nucleotide changes are localised to a small section and lead to amino acid differences on the C-terminal side of the intrachain thioester bond about the middle of the pro-C4 molecule. Though synthesised as a single peptide chain of 1722 residues C4 is processed before secretion to give a three-chain structure, α-chain (767 residues) β-chain (656 residues) and γ-chain (291 residues) (Belt *et al.*, 1984). On activation by C$\bar{1}$ in the complement cascade, a reactive acyl group is released from the thioester bond near the centre of the α-chain to form a covalent bond with the activating substances. Another unusual feature is that the different forms of C4 show big differences in several biological activities and this offers the opportunity of correlating the small changes of amino acid sequence with the different activities.

In the activation scheme, C4 is activated by C$\bar{1}$, forms a covalent bond with antibody and antigen, interacts with C2 to form C3 convertase and with C3 to form C5 convertase. It is inactivated by factor I with the co-factor C4 binding protein. Differences in any of these reactions could alter the haemolytic activity of C4. The several-fold lower activity of C4-A than C4-B allotypes has been shown to be due to differences in covalent bond formation with antibody-coated red cells. This is due to very large differences in the rates of reaction between C4-A and C4-B allotypes with amino groups (in glycine), and with hydroxyl groups (in glycerol) (Law *et al.*, 1984a; Isenman and Young, 1984).

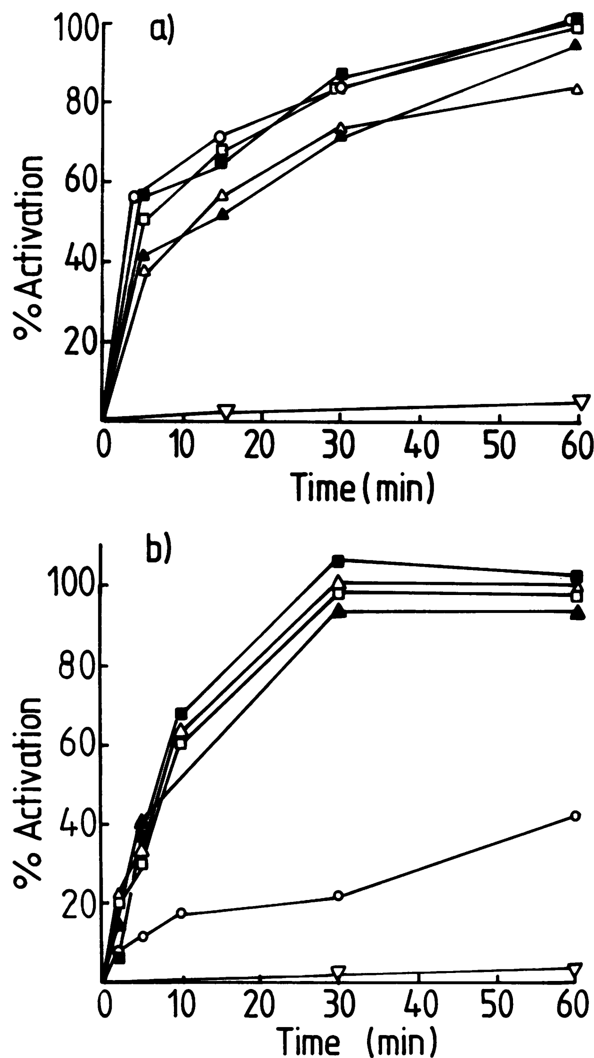


**Fig. 4.** Catalytic activity of **(a)** C3 convertase and **(b)** C5 convertase made from different C4 allotypes. EAC14 cells were prepared using C4-A3 (□); C4-A4 (△); C4-A6 (○); C4-B1 (■); C4-B2 (▲); and a control with no C4 (▽). The degree of hydrolysis of C3 (a) and C5 (b) with times were determined as described in Materials and methods.

There are differences in activity between some alleles of the same locus, notably in C4-A6 which shows little or no activity when measured in the haemolytic overlay assay (Teisberg *et al.*, 1980; O'Neill *et al.*, 1980). Standard assay of purified C4-A6 has shown it to have ~10% of the activity of the other C4-A allotypes (Table I). It seemed likely that this would be due to lower covalent binding to the antibody-coated red cells, but no difference was found in this reaction between C4-A3 and C4-A6 though both were less active than C4-B1, as expected (Figure 2). When the pattern of red cell surface proteins to which C4 binds was examined in SDS-PAGE, that for the C4-B1 allotype differed from the C4-A allotypes (Isenman and Young, 1985) but there was no difference between C4-A6 and C4-A3 (Figure

3). Qualitatively as well as quantitatively the C4-A allotypes had the same reactivity with sensitised red cells. Similarly, binding to amino groups and hydroxyl groups showed no differences between the C4-A allotypes though there are very big differences between the products of the two loci (Tables II and III).

When rates of activation of C4 by C1s and inactivation by factor I and C4 binding protein were measured, no differences were found between the various allotypes (Table IV). The catalytic activity of the C3 convertase formed using different allotypes was also very similar but the C5 convertase formed from C4-A6 showed a much lower activity than when formed from the other allotypes (Figure 4). Measurement of initial rates of hydrolysis of C5 with this technique are not accurate but the proteolytic digestion of C5 by C5 convertase using the C4-A6 allotype is probably < 1/5th that of the convertase with other C4 allotypes. Activated $\bar{C5}$ reacts spontaneously with C6 and C7 and then with C8 and C9, the five proteins together forming the cytolytic complex in the cell membrane. It is probable that the rate of C5 activation will determine the amount of lytic complex formed as the duration of complement activation is limited by inhibitors and inactivators at every step in the cascade. If this is correct, the low activity of C5 convertase formed with C4-A6 explains the low haemolytic activity of this allotype.

The difference in binding activity of C4-A and C4-B allotypes appears to depend on changes in six amino acid residues C-terminal of the thioester bond which probably determine the environment of the reactive acyl group either directly or indirectly due to conformational changes induced by the changes. If only one or two amino acid residue changes distinguish C4-A6 from other C4-A allotypes, as seems likely from the sequence comparison of other alleles (Belt *et al.*, 1985), they should indicate the critical section of peptide chain which influences C5 convertase activity. C5 convertase is formed from C4 bound to the antibody-coated red cell with which $\bar{C2}$ interacts non-covalently to give C3 convertase and then C3 binds covalently to give the C5 convertase. As the C3 convertase activity is the same for all alleles, the variant section of peptide chain in C4-A6 is likely to reduce the C5 convertase activity at the final stage in its assembly. This is the covalent binding of C3 to the C3 convertase and it might lead to decreased stability or perhaps affect directly the interaction of the substrate, C5, with the proteolytic site in the activated $\bar{C2}$ subunit of this complex enzyme.

While the activity of the C4-A6 allotype is strikingly lower than that of other allotypes, the activity of others within each of the C4-A series or C4-B series appears to be similar. However, the comparisons are only of haemolytic activity and *in vivo* the complement system is responsible for the destruction of many different pathogens with a very wide range of surface structures. It is also essential for the dissolution of immune aggregates and their removal by binding to cells through the C4- and C3-receptors. The various C4 allotypes may show other differences in activity *in vivo*, not apparent in the *in vitro* haemolytic assay. These differences could determine the efficiency of destruction and elimination of different pathogens. The presence of particular C4 allotypes in an individual's blood will therefore influence his resistance to particular diseases and perhaps also his susceptibility to tissue damage by autoimmune reactions.

## Materials and methods

*Monoclonal Antibodies*

Monoclonal antibody to C4 (L003) in the form of ascites fluid were purchased from The Commonwealth Serum Laboratories, Parkville, Victoria, Australia. The IgG fraction was purified (Wilkinson, 1969) to give a yield of ~35 mg of IgG from 5 ml of ascites fluid. An affinity column was prepared by coupling 10 mg of monoclonal antibody to 1.5 g of CNBr-activated Sepharose (Pharmacia) following the manufacturer's procedure. Binding efficiency was >95%.

*Preparation of C4 allotypes*

10 ml of plasma containing 10 mM EDTA, pH 7.4, to which diisopropylfluorophosphate (DFP) was added to 10 mM, were loaded on to the L003 monoclonal antibody column pre-equilibrated with a buffer containing 50 mM Tris, 50 mM Na-phosphate, 12.5 mM Na-tetraborate, 2.5 mM EDTA, 0.1 mM phenylmethylsulphonyl-fluoride (PMSF) and 0.02% Na-azide at pH 7.0. The column was washed with buffer until the $OD_{280}$ of the eluate approached zero. C4 was eluted with a linear gradient from 20 ml reservoirs of buffer containing 100 mM Tris, 100 mM Na-phosphate, 25 mM Na-tetraborate, 5 mM EDTA, 0.2 mM PMSF and 0.02% Na-azide, one at pH 8.5 and the other at pH 11.5. Eluate fractions were scanned by SDS-PAGE in gels with an acrylamide:bisacrylamide ratio of 1:0.006 (Roos *et al.*, 1982) and those containing C4-A or C4-B were pooled and dialysed into 25 mM Na-phosphate, 25 mM NaCl, 0.5 mM EDTA, 0.2 mM PMSF, pH 7.5. The proteins were concentrated by loading on to a 1 ml column of DEAE-Sephacel (Pharmacia) equilibrated in the same buffer. Elution was with 3 ml of the same buffer containing 500 mM NaCl. The protein was dialysed into 10 mM Na-phosphate, 125 mM NaCl, 0.5 mM EDTA, pH 7.5, and stored at 4°C.

*Preparation of other complement proteins*

Other complement proteins were prepared according to published procedures: human C1 and $C\bar{1}s$ (Gigli *et al.*, 1976), C2 (Kerr, 1981), C3 and C5 (Hammer *et al.*, 1981), factor I (Hsiung *et al.*, 1982), and C4 binding protein (Reid and Gagnon, 1982). C4 and C5 were iodinated using Iodobeads (Pierce) according to the manufacturer's instruction.

Except for the determination of different C4 allotypes during fractionation on L003 column (see above), all SDS-PAGE were done in the Laemmli system (Laemmli, 1970). Gels were stained according to Fairbanks *et al.* (1971).

*Haemolytic assays*

The buffer used throughout was $DGVB^{2+}$, dextrose gelatin veronal buffer with $Ca^{2+}$ and $Mg^{2+}$ (Borsos and Rapp, 1967). Erythrocytes coated with IgM (EA) were prepared (Mayer, 1961) and EA-bearing C1 (EAC1) using human C1 according to Borsos and Rapp (1967).

C4 haemolytic assays using guinea pig C4-deficient serum were performed as described by Law *et al.* (1980). C4 haemolytic assays were also performed using human R4 reagent prepared by passing human serum through rabbit anti-human C4 $Fab'_2$ Sepharose (Law *et al.*, 1984a), and by the method of Gigli *et al.* (1977), using EAC1 cells, purified guinea-pig C2 and guinea-pig C-EDTA to supply the terminal components.

Cleavage of C4 by $C\bar{1}s$ and C4b by factor I and C4 binding protein were performed as described previously (Law *et al.*, 1984a).

*C4 binding to red cells*

C4-A3, C4-A6 and C4-B1 were labelled with $^{125}I$ to ~$5 \times 10^5$ c.p.m./μg without loss of haemolytic activity. 400 μl of various concentrations of $[^{125}I]C4$ (7.5 μg/ml − 30 ng/ml) in $DGVB^{2+}$ were added to $4 \times 10^8$ EAC1. The mixtures were incubated for 15 min at 37°C. The cells were washed three times with phosphate-buffered saline and transferred to fresh tubes and counted. The binding of $[^{125}I]C4$ to EA cells was measured as control.

Membrane polypeptides of EAC14 cells bearing $[^{125}I]C4b$ were prepared and analysed by SDS-PAGE (Law and Levine, 1977). The gels were stained before autoradiography.

*Binding of C4 to glycine and glycerol*

The covalent binding activity of C4 to $[2\text{-}^3H]$glycine (100 mCi/mmol) and $[2\text{-}^3H]$glycerol (200 mCi/mmol) was determined using $C\bar{1}s$ to activate C4 (Law *et al.*, 1984b). Radioactive glycine (15 Ci/mol) and glycerol (200 mCi/mmol) were purchased from the Amersham International, UK and New England Nuclear, respectively.

*C3 convertase*

EAC14 cells were prepared using human C1 and purified C4 allotypes. For C4-A allotypes, 2 μg of C4 and for C4-B allotypes 0.7 μg of C4 were incubated with $1 \times 10^8$ EAC1 cells in $DGVB^{2+}$ at 37°C for 15 min. In each case, ~3000 molecules per cell were bound.

Tubes containing $1 \times 10^7$ EAC14 cells, 12 μg human C3 and 200 units of human C2 were incubated at 37°C for various times. The supernatant was then reduced and alkylated and run on SDS-PAGE. The gel were stained with Coomassie blue. Cleavage of C3 α-chain to C3 α'-chain was quantitated by laser densitometry.

*C5 convertase*

EAC14 cells were converted to EAC143 by incubation of $1 \times 10^8$ cells with 120 μg C3 and 2000 units of human C2 for 30 min at 37°C. $1 \times 10^7$ EAC143 cells, 0.5 μg $[^{125}I]C5$ and 200 units of human C2 in $DGVB^{2+}$ were incubated at 37°C for various times. Because of high non-specific binding of C5 and C5b

to the tube and cells it was necessary to load the whole mixture on to gels after urea-SDS solubilisation, reduction and alkylation. After staining with Coomassie blue gels were dried and autoradiographed. Cleavage of the C5$\alpha$-chain to the C5$\alpha'$-chain was measured by scanning the autoradiographs.

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