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The low C5 convertase activity of the C4A6 allotype of human complement component C4

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We have compared the C5-convertase-forming ability of different C4 allotypes, including the C4A6 allotype, which has low haemolytic activity and which has previously been shown to be defective in C5-convertase formation. Recent studies suggest that C4 plays two roles in the formation of the C5 convertase from the C3 convertase. Firstly, C4b acts as the binding site for C3 which, upon cleavage by C2, forms a covalent linkage with the C4b. Secondly, C4b with covalently attached C3b serves to form a high-affinity binding site for C5. Purified allotypes C4A3, C4B1 and C4A6 were used to compare these two activities of C4. Covalently linked C4b–C3b complexes were formed on sheep erythrocytes with similar efficiency by using C4A3 and C4B1, indicating that the two isotypes behave similarly as acceptors for covalent attachment of C3b. C4A6 showed normal efficiency in this function. However, cells bearing C4b–C3b complexes made from C4A6 contained only a small number of high-affinity binding sites for C5. Therefore a lack of binding of C5 to the C4b–C3b complexes is the reason for the inefficient formation of C5 convertase by C4A6. The small number of high-affinity binding sites created, when C4A6 was used, were tested for inhibition by anti-C3 and anti-C4. Anti-C4 did not inhibit C5 binding, whereas anti-C3 did. This suggests that the sites created when C4A6 is used to make C3 convertase may be C3b–C3b dimers, and hence the low haemolytic activity of C4A6 results from the creation of low numbers of alternative-pathway C5-convertase sites.

INTRODUCTION

The fourth component of complement (C4) is a precursor of a subunit of the complex enzymes C3 convertase, C4b2a and C5 convertase, C4b2a3b, of the classical pathway (Reid & Porter, 1981; Müller-Eberhard, 1988). C4 has an intramolecular thioester which is formed between a thiol group of Cys-991 and a carbonyl group of Gln-994 in the C4d region (Campbell *et al.*, 1981; Tack, 1983). Upon activation of C4 by $\overline{C1}s$ the thioester becomes reactive and forms a covalent linkage with an amino or a hydroxy group on the target surface (Law *et al.*, 1984; Isenman & Young, 1984). C2 becomes bound to C4b and is in turn cleaved by $\overline{C1}s$ (Reid & Porter, 1981; Müller-Eberhard, 1988). The C3 convertase, C4b2a, thus generated cleaves multiple molecules of C3 to C3a and nascent C3b (Reid & Porter, 1981). C3 also contains a thioester bond, and through this a nascent C3b molecule becomes covalently bound to C4b and the C5 convertase, C4b2a3b, is generated (Takata *et al.*, 1987). The C5 convertase has a high affinity for C5, the K_a being 2.1×10^8 M^{-1} (Takata *et al.*, 1987). Previous studies have shown that C3b has binding affinity for C5 (Isenman *et al.*, 1980; DiScipio, 1981). However, this affinity is 50 times lower than that between C5 and the convertase. Since there is evidence that C4b also plays a role in the association of the convertase with C5 (Takata *et al.*, 1987), it seems likely that bivalent association accounts for the high-affinity binding of C5 to the convertase. Therefore, C4b plays two roles in the formation of C5 convertase from C3 convertase. First, C4b acts as an acceptor for the covalent attachment of the third subunit C3b. Second, C4b serves to form part of the high-affinity binding site for C5.

Two isotypes, C4A and C4B, are known for C4 (O'Neill *et al.*, 1978) which are coded by closely linked genes within the HLA class III region (Campbell *et al.*, 1988). Although they show over 99% amino acid sequence identity (Belt *et al.*, 1985; Yu *et al.*, 1986), the reactivities of their thioesters are different. An amide bond is preferentially formed by activated C4A, and an ester bond is preferentially formed by activated C4B (Law *et al.*, 1984; Isenman & Young, 1984). Both C4 genes are highly polymorphic and, for each isotype, many allotypes are known (Mauff *et al.*, 1983). C4A3 and C4B1 are the most common allotypes of A and B isotypes respectively (Rittner & Schneider, 1988).

One allotype of C4A, namely C4A6, is defective in that it has very low haemolytic activity (Teisberg *et al.*, 1980). Previous studies have shown that the formation of a C3 convertase containing C4A6 is normal, but that that of the C5 convertase is not (Dodds *et al.*, 1985).

In the present investigation we have compared the activity of C4A6 with those of the normal allotypes C4A3 and C4B1 to determine which step in the formation of C5 convertase is defective in C4A6. Our results show that C4b made from C4A6 acts normally as an acceptor for the covalent binding of C3b, but that the resulting

Abbreviations used: DGVB, Dextrose/gelatin/veronal buffer (for composition, see the text); SDS/PAGE, SDS/polyacrylamide-gel electrophoresis; the nomenclature of complement components is as recommended by the World Health Organization (1968, 1981); ^{ox}C2, human C2 oxidized with iodine to enhance its stability; complement component fragments are denoted by a lower-case letter e.g. C4b, C2a; complement component C4 isotypes are denoted by an upper-case letter e.g. C4A, C4B; C4 allotypes are denoted by a number, e.g. C4B1, C4A6 (Mauff *et al.*, 1983); E, sheep erythrocytes; A, IgM antibody to sheep erythrocyte stromata; C, complement component (e.g. EAC14 are sensitized sheep erythrocytes bearing C1 and C4).

C4b–C3b complex does not contain a high-affinity binding site for C5. Analysis of the primary structure of C4A6 should be informative in determining the amino acid residues involved in the C5-binding site of the C5 convertase.

MATERIALS AND METHODS

Buffers

The buffer used for all experiments was iso-osmotic veronal buffer, pH 7.4, containing 2.5 mM-sodium barbitone, 72.7 mM-NaCl, 2.5% dextrose, 0.1% gelatin, 0.15 mM-$CaCl_2$ and 1 mM-$MgCl_2$ (DGVB). Sample buffer for SDS/PAGE contained 125 mM-Tris/HCl, pH 6.8, 4% (w/v) SDS, 10% (v/v) glycerol and 0.005% Bromophenol Blue.

Complement components

C4 allotypes were purified from the plasma of donors of known C4 phenotype. Two donors with C4A6B1 were used as a source of C4A6. Plasma from a donor homozygous for C4A3 and B1 was used to prepare C4A3 and C4B1. Purification of C4 was performed by using a monoclonal antibody with different affinities for the two isotypes (Dodds *et al.*, 1985). Briefly, the IgG of the anti-C4 monoclonal antibody L003 was coupled to CNBr-activated Sepharose 4B (Pharmacia) at 5 mg/ml of packed beads and plasma was passed through the affinity column. Bound C4 was eluted by a pH gradient. C4A and C4B were eluted sequentially with rising pH. Each isotype fraction was subjected to an additional affinity-chromatographic separation to reduce cross-contamination to a very low level. C4A6 was affinity-purified for a third time to ensure negligible contamination with C4B1. The affinity-purified C4 was concentrated, and trace contaminants removed by ion-exchange chromatography on Mono Q (Pharmacia) (Dodds & Law, 1988). The purity of each allotype preparation was confirmed by SDS/PAGE, which can distinguish between the two isotypes (Roos *et al.*, 1982). Guinea-pig C1 (Nelson *et al.*, 1966) and C2 (Tamura, 1970) and human C2 (Kerr, 1981), C3 and C5 (Tack & Prahl, 1976) were purified as described in the references cited. Human C2 was oxidized (^{oxy}C2) as described by Polley & Müller-Eberhard (1967).

C4, C3 and C5 were radiolabelled with ^{125}I using Iodogen (Pierce Chemical Co., Rockford, IL, U.S.A.)-coated test tubes (Fraker & Speck, 1978). The preparations had specific radioactivities between 2×10^6 and 6×10^6 c.p.m./μg of protein and were fully active after iodination.

Red-cell-haemolytic intermediates

Antibody-sensitized sheep erythrocytes (EA) bearing C1 (EAC1) were prepared by incubation of EA cells (10^8/ml) with guinea-pig C1 (200 units/ml) for 15 min at 30 °C (Nelson *et al.*, 1966). EAC14 cells were prepared by incubating EAC1 cells (10^8/ml) with an equal volume of C4 (either radiolabelled or unlabelled) at a concentration of 0.2–10 μg/ml for 30 min at 30 °C. C4A3 and C4A6 were used at three times the concentration of C4B1 to give approximately equal numbers of C4b molecules per cell (Dodds *et al.*, 1985). EAC142 cells were prepared by incubating EAC14 cells (10^8/ml) with an equal volume containing 400 units of guinea-pig C2 or human ^{oxy}C2/ml for 10 min at 30 °C (Nelson *et al.*, 1966). EAC1423 cells were prepared by incubating EAC142 cells (10^8/ml) with an equal volume of human C3 (either radiolabelled or unlabelled) at a concentration of 1–30 μg/ml for 20 min at 30 °C. The amounts of C4b and C3b deposited on the cells were determined from the uptake of radiolabelled C4 and C3. EAC143 cells were prepared by incubating EAC1423 cells bearing guinea-pig C2 in DGVB for 60 min at 37 °C.

SDS/PAGE

SDS/PAGE was performed as described by Laemmli (1970). To check the purity of C4 allotype preparations, a system described by Roos *et al.* (1982) was used. The molecular-mass marker proteins used were IgM (900 kDa), C4-binding protein (560 kDa) and factor H (150 kDa).

Determination of the percentage covalent binding of C4b to C3b on EAC1423 cells

EAC1423 cells bearing radiolabelled C4b were solubilized in SDS/PAGE sample buffer and subjected to SDS/PAGE on 4.5% gels under non-reducing conditions, followed by autoradiography. The intensities of the bands of C4b–C3b complexes and C4b monomers on the autoradiogram were measured with a densitometer (DU-83; Beckman Instruments, Fullerton, CA, U.S.A.). The percentage covalent binding of C4b to C3b was calculated as 100 × (the intensity of the C4b–C3b-complex band)/(the total intensity of the C4b-monomer band plus the C4b–C3b-complex band).

Assay of binding of C5 to EAC1423 or EAC143 cells and determination of the number of C5-binding sites

Samples of cell suspension (10^8/ml) were incubated with equal volumes containing increasing concentrations of radiolabelled C5 (125–2000 ng/ml) in DGVB for 60 min at 30 °C. Duplicate 40 μl samples from each reaction mixture were layered over 300 μl of a mixture of dibutyl phthalate and dinonyl phthalate (Nakarai Chemicals, Kyoto, Japan) (7:3, v/v). After centrifugation at 8000 ***g*** for 3 min at 4 °C, the tubes were cut just above the cell pellets and the radioactivity of the bound C5 in the pellets was measured. The amount of free C5 was calculated by subtracting the radioactivity in the pellets from the total radioactivity. Data obtained from the binding assays were analysed by a Scatchard (1949) plot to determine the number of C5-binding sites and the association constants.

Inhibition of C5 binding to EAC1423 cells by anti-C4 and anti-C3 antibodies

EAC1423 cells (10^8/ml in DGVB) were mixed with IgG (0–45 μg/ml in DGVB) from rabbit anti-C4 or anti-C3 antiserum, and the mixtures were incubated at 4 °C for 10 min. An equal volume of radiolabelled C5 (1200 ng/ml in DGVB) was then added to the mixtures and, after incubation at 30 °C for 60 min, the amount of C5 bound to the antibody-treated cells was determined as described above.

RESULTS

Demonstration of the formation of covalently linked C4b–C3b complexes using C4A6

The efficiency of the formation of covalently linked C4b–C3b complexes using C4A6 was compared with that of those prepared using the normal allotypes C4A3

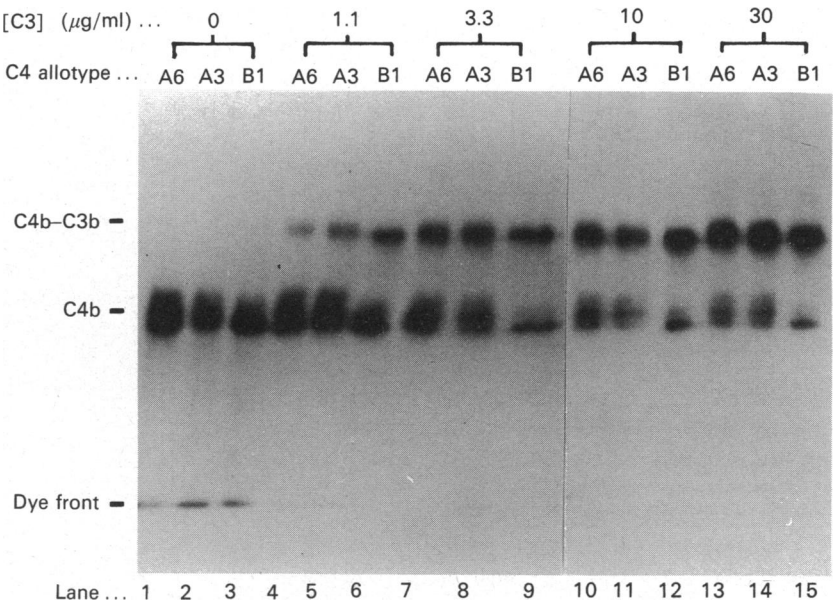

Fig. 1. SDS/PAGE analysis of formation of C4b–C3b complexes with different C4 allotypes

EAC14^{oxy}2 cells (3.3×10^7/ml) bearing radiolabelled C4b (~ 1500 molecules/cell) from different allotypes were incubated with increasing amounts of C3 (0–30 μg/ml) in DGVB at 30 °C for 20 min. The cells were then washed with DGVB and hypo-osmotically lysed in 15 mM-Tris/HCl, pH 6.9. The resulting ghosts were dissolved in non-reducing SDS/PAGE sample buffer and subjected to SDS/4.5% PAGE, followed by autoradiography.

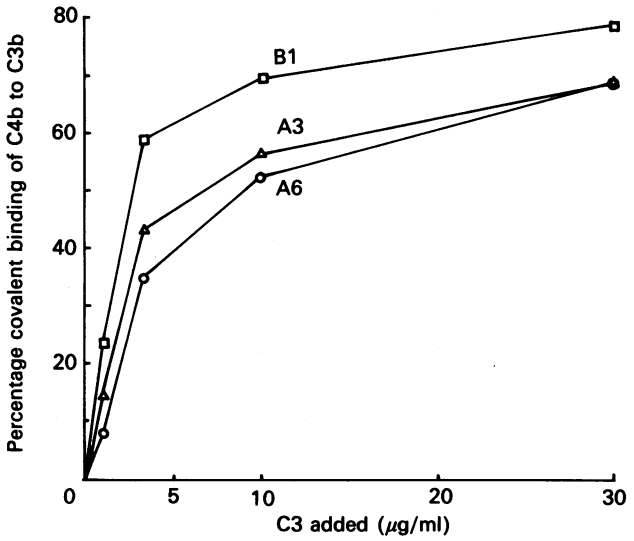

Fig. 2. Formation of C4b–C3b complexes with different C4 allotypes

The 'film' shown in Fig. 1 was scanned with a densitometer and the percentage covalent binding of C4b to C3b determined. The percent covalent binding was plotted as a function of the amount of C3 offered to the EAC14^{oxy}2 cells. The EAC14^{oxy}2 cells were prepared using C4B1 (□), C4A3 (△) or C4A6 (○).

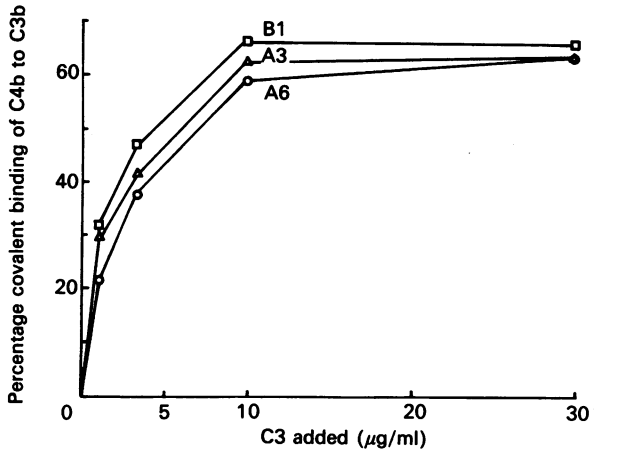

Fig. 3. Formation of C4b–C3b complexes with different C4 allotypes

An experiment similar to that shown in Figs. 1 and 2 was performed using unoxidized C2 instead of oxidized C2. The EAC142 were prepared using C4B1 (□), C4A3 (△) or C4A6 (○).

and C4B1. Three groups of EAC14^{oxy}2 cells, each bearing approximately the same number of C4b molecules of each allotype, were prepared with radiolabelled C4. The cells were incubated with increasing amounts of C3 to allow covalent attachment of C3b to the cells. The resulting EAC14^{oxy}23 cells were subjected to SDS/PAGE followed by autoradiography to determine the extent of the formation of C4b–C3b complexes. As shown in Fig. 1, with increasing amounts of C3 offered, the bands of C4b at 200 kDa shifted to bands of C4b–C3b complexes at 400 kDa. C4b–C3b complexes were formed efficiently with each of the C4 allotypes tested including C4A6. For quantitative estimation, the film was scanned with a densitometer and the percentage covalent binding of C4b to C3b was determined. As shown in Fig. 2, C4b–C3b complexes were formed efficiently with each C4 allotype, more than 60% of C4b being complexed with C3b at the

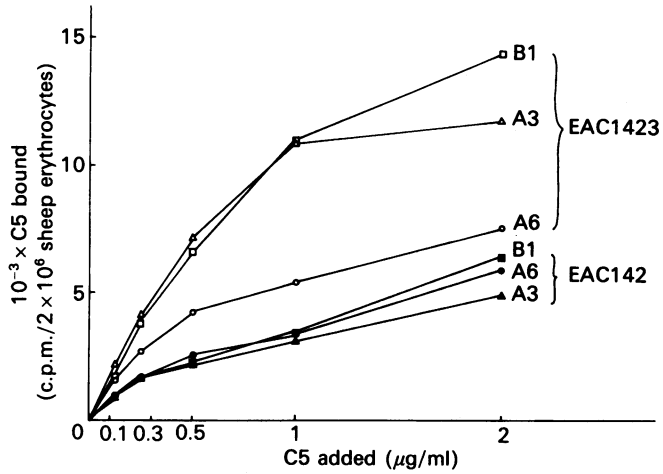

Fig. 4. Binding of C5 to EAC14^{oxy}23 cells bearing different C4 allotypes

EAC14^{oxy}23 cells (open symbols) and EAC14^{oxy}2 cells (closed symbols) bearing different C4 allotypes (5×10^7/ml and 10000 molecules of C4b/cell) were incubated with increasing amounts of radiolabelled C5 (0.125–2.0 μg/ml). The amount of C5 bound is plotted as a function of the amount of C5 added. The cells were prepared using C4B1 (□, ■), C4A3 (△, ▲) or C4A6 (○, ●).

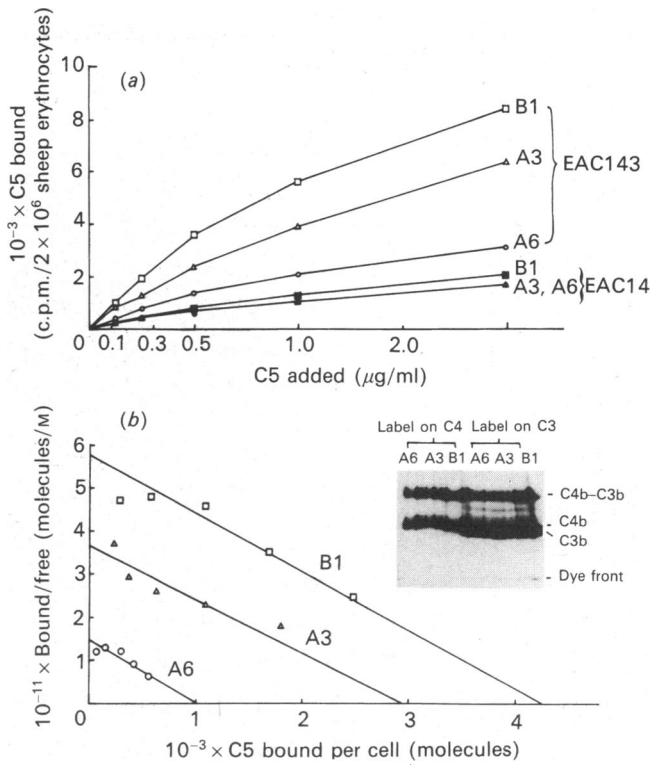

Fig. 5. Binding of C5 to EAC143 cells bearing different C4 allotypes

EAC143 cells bearing approximately the same numbers of C4b–C3b complexes made from different C4 allotypes were prepared. The amounts of C4b–C3b complexes on the cells were confirmed by making parallel cell preparations with radiolabelled C4 or C3 and analysing them by SDS/PAGE and autoradiography. The result is shown in the inset. The EAC143 cells (open symbols) and EAC14 cells (closed symbols) as a control (10^8/ml) bearing unlabelled C4b (~ 10000 molecules/cell) and unlabelled C3b (~ 20000 molecules/cell) were incubated with radiolabelled C5 (0.125–2.0 μg/ml) for 60 min at 30 °C, and the amount of C5 bound to the cells was determined. (*a*) C5 bound to the cells was plotted as a function of the amount of C5 added. The cells were prepared using C4B1 (□, ■), C4A3 (△, ▲) or C4A6 (○, ●). (*b*) Scatchard plot of the binding data shown in (*a*). Specific binding was determined by subtracting the C5 bound to EAC14 cells from that bound to EAC143 cells and subjected to Scatchard analysis. Association constants for C5 binding to EAC143 cells made with C4B1 (□), C4A3 (△) and C4A6 (○) were 1.3×10^8 M^{-1}, 1.3×10^8 M^{-1} and 1.5×10^8 M^{-1} respectively.

highest C3 concentration. C4A6 purified from a second donor was also tested and the same result was obtained (result not shown). In the next experiment, unoxidized C2 was used instead of oxidized C2 to confirm the above result. Again efficient complex-formation was observed with each C4 allotypes (Fig. 3). When oxidized C2 was used, the efficiency of complex formation at low C3 concentrations using C4A6 was lower than that using C4A3 (Fig. 2). A less prominent difference was also observed when unoxidized C2 was used (Fig. 3). However, the extent of difference in the efficiency of complex-formation does not seem to account for 15-fold lower haemolytic activity of C4A6 (Dodds *et al.*, 1986*a*). These results suggest that the acceptor site for the covalent binding of C3b is normal on C4b derived from C4A6.

Inefficient binding of C5 to C4b–C3b complexes made from C4A6

We next tested whether C5 bound with high affinity to the C4b–C3b complexes made from C4A6. Three groups of EAC14^{oxy}2 cells bearing approximately equal numbers of C4b molecules of the C4B1, C4A3 or C4A6 allotypes were incubated with a constant amount of C3 to convert them into EAC14^{oxy}23 cells bearing comparable amounts of C4b–C3b complexes. These cells were incubated with radiolabelled C5 and the amount of C5 bound was determined (Fig. 4). The binding of C5 to EAC14^{oxy}23 cells made from C4A3 and C4B1 was comparable. Significantly less C5 bound to the cells made from C4A6, suggesting that inefficient binding of C5 may be the basis of the low activity of C4A6 in C5 convertase formation. A similar result was obtained using C4A6 prepared from another donor (results not shown).

To avoid any effect of C5 cleavage by C2, EAC143 cells were used in the next experiment. Three groups of EAC1423 cells, each bearing approximately the same number of C4b molecules of each allotype, were prepared and incubated in DGVB at 37 °C for 1 h to allow decay dissociation of C2a. Parallel sets of cells were prepared with either radiolabelled C4 or radiolabelled C3 and they were used for SDS/PAGE analysis to confirm that comparable amounts of C4b–C3b complexes were present on these cells. As shown in the inset to Fig. 5, the three types of cells bore approximately the same amounts of C4b–C3b complexes. EAC143 cells bearing unlabelled C4b and C3b were then incubated with radiolabelled C5 to measure C5 binding. Again, EAC143 cells made from C4A6 did not bind C5 efficiently (Fig. 5*a*), confirming that inefficient binding of C5 to the C4b–C3b complexes is the reason for the low activity of C4A6.

Although the amount of binding of C5 to EAC1423 or

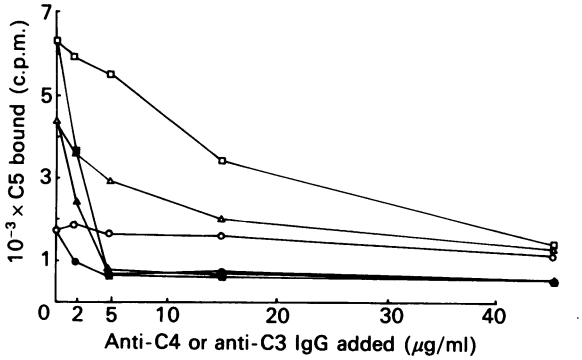

Fig. 6. Inhibition of C5 binding to EAC1423 cells by anti-C3 and anti-C4 antibodies

EAC1423 cells (~ 10000 molecules C4b/cell and ~ 20000 molecules C3b/cell) made with C4B1 (□, ■), C4A3 (△, ▲) or C4A6 (○, ●) were incubated with anti-C4 (open symbols) or anti-C3 (closed symbols) IgG (0–45 μg/ml) at 4 °C for 10 min before the addition of radiolabelled C5 (80000 c.p.m.). After incubation at 30 °C for 60 min, the amount of C5 bound to the antibody-treated cells was determined.

EAC143 cells made from C4A6 was very low, it was not zero (Figs. 4 and 5). Scatchard analysis of the binding data shows that there are high-affinity (~ 1.5×10^8 M^{-1}) C5-binding sites on the cells made from all allotypes, but that the number of sites is lower on cells made with C4A6 than on the cells made with C4A3 or C4B1 (Fig. 5*b*).

We used anti-C3 and anti-C4 antibodies to determine whether it was possible to inhibit the binding of C5 to EAC1423 cells made using the different allotypes. As shown in Fig. 6, the addition of anti-C4 (open symbols) could inhibit the binding of C5 to cells prepared using C4A3 or C4B1, reducing the amount of C5 bound to the level seen on cells prepared with C4A6. However, anti-C4 had little effect on the binding of C5 to cells prepared with C4A6. When anti-C3 was used (closed symbols), C5 binding to all three cell types was reduced to a very low level that probably represents non-specific binding. These results suggest that the C4A6 is not involved in the high-affinity binding sites found on cells prepared using this allotype, but that C3 is. The cells prepared by using C4A3 and C4B1 also appear to have high-affinity binding sites which are inhibitable by anti-C3 but not by anti-C4. The most likely explanation for these observations is that a small number of C3b–C3b dimers are generated on the surface by the classical-pathway C3 convertase and that these form alternative-pathway high-affinity C5-binding sites.

DISCUSSION

In a previous study it was shown that the C4A6 allotype has a low, but significant, haemolytic activity, approx. 15-fold lower than that of C4A3 (Dodds *et al.*, 1986*a*). It was found that this lower activity was not due to differences in the rate of cleavage of C4A6 by $C\bar{1}s$, the rate of inactivation of C4A6b by C4-binding protein and factor I, the stability of the protein, the covalent binding reaction with the cell surface or an inability to form C3 convertase. The sole difference between C4A6 and C4A3 was in their ability to form an efficient C5 convertase (Dodds *et al.*, 1985).

Recent studies have shown that formation of the classical-pathway C5 convertase requires the covalent attachment of C3b to the α'-chain of the C4b molecule of the classical pathway C3 convertase, C4b2a (Takata *et al.*, 1987). The formation of the C4b–C3b complexes leads to the generation of high-affinity binding sites for C5 which are necessary for the generation of C5 convertase activity. The C5 convertase of the alternative pathway is similarly formed by covalent binding of C3b to the C3b of the alternative pathway C3 convertase, C3bBb (Kinoshita *et al.*, 1988). Here we have attempted to establish which step in C5-convertase generation is inefficient when C4A6 is used. There appeared to be four possible stages at which C4A6 could be defective: (1) The amino acid residue to which the nascent C3b binds covalently could be missing or altered; (2) the binding site(s) for C5 on the C4b–C3b complex could be defective; (3) the binding site for C2a could be different such that the enzymic activity of the C5 convertase was lowered; (4) the rate of release of activated C5b from the enzyme could be lower.

The results described here indicate that the most likely step at which C4A6 is deficient is at stage 2, namely the binding of C5 to the C4b–C3b complex. We have shown that covalent attachment of C3b to the C4b of the C3 convertase is efficient with all three allotypes tested (Figs. 1–3). When C5 binding to $EAC14^{oxy}23$ was measured, the binding to cells bearing C4A6 was found to be considerably lower than that seen with C4A3 or C4B1 (Fig. 4). Binding experiments were also performed with EAC143 cells after decay of C2a from EAC1423. C4A6 binding was lower than the binding of C4A3 or C4B1 (Fig. 5*a*). In this experiment, approx. 30% less C5 bound to cells bearing C4A3 than to those bearing C4B1. This result was consistently obtained over four repetitions of the same experiment; we have no explanation for this observation. The fact that the binding of C5 by C4A6 was similar whether C2a was present or absent indicates that a difference in the enzymic properties of the C5 convertase formed with C4A6 is probably not responsible for its inactivity, hence also release of C5b is not involved. Scatchard analysis (Fig. 5*b*) indicated that the difference in the binding of C5 to the cells prepared with different C4 allotypes probably resulted from a difference in the number of binding sites per cell, whereas the association constants remained similar. This was a little surprising, as it might be expected that the defect in C4A6 would lead to a lowering of affinity of C5 for the C4b–C3b complex, whereas the number of sites per cell would remain constant.

One possible explanation for these observations would be contamination of the C4A6 protein with C4B1, since the C4A6 was prepared by affinity chromatography of plasma from donors who expressed both C4 types. It had previously been shown that, after a single run on the L003 column, contamination of C4A with C4B is approx. 0.25% (Dodds *et al.*, 1986*a*). For the experiments described here, C4A6 was re-purified on the L003 monoclonal-antibody column three times, and cross-contamination with C4B1 should therefore be negligible.

A likely explanation for the apparent anomaly was obtained from experiments in which we inhibited C5 binding to the C4b–C3b complexes with either anti-C4 or anti-C3. Treatment of cells, prepared with the different

allotypes, with anti-C4 (Fig. 6, open symbols) led to an inhibition of C5 binding to the cells prepared with C4A3 and C4B1, but had no effect on cells prepared with C4A6. The antibody used for these experiments was rabbit polyclonal anti-(human C4). Although it is possible that a very small amount of isotype- or allotype-specific antibody is present, most of the antibody will be directed against determinants common to all of the C4 types used. It is therefore unlikely that the anti-C4 would show differential binding to the different allotypes. This suggests that C4A6 may play no role in the high-affinity C5-binding sites on cells prepared with this allotype. C5 binding to cells prepared with C4A3 or C4B1 was reduced, by treatment with anti-C4, to a level similar to that seen with cells prepared with C4A6. This suggests that, although most of the high-affinity binding sites on these cells involve C4, some may be C4-independent. Treatment of all of the cell types with anti-C3 (Fig. 6, closed symbols) reduced C5 binding to a level below that possible with anti-C4. Anti-C3 would be expected to inhibit C5 binding to the C4b–C3b complexes, but also to complexes containing only C3b, that is, the alternative-pathway C5 convertase containing C3b–C3b complexes, which would not be affected by anti-C4. From these observations it seems likely that C4A6 is completely deficient in its ability to form high-affinity C5-binding sites, and hence C5 convertase. The high-affinity sites created when C4A6 is used in the C3 convertase probably result entirely from the formation of C3b dimers and hence amplification or alternative-pathway C5 convertase sites. The C3 convertases prepared using C4A3 or C4B1 also appear to generate a similar number of C3b dimers, so creating high-affinity binding sites which cannot be inhibited by anti-C4.

These observations also provide a satisfactory explanation for the low haemolytic activity of C4A6 when measured using C4-deficient guinea-pig serum. When C3 is activated by the C4b2a enzyme on the cell surface, not only C4b–C3b dimers, but also, to a lesser extent, C3b–C3b dimers, were formed. The C4b–C3b complex, if made with C4A6, may not be active in forming the classical-pathway C5 convertase. However, the C3b–C3b complex is fully active, as the non-catalytic component of the alternative-pathway C5 convertase, to catalyse the formation of functional membrane-attack complexes.

Further work is necessary to determine the amino acid residue(s) which are altered in C4A6 in comparison to C4A3. This should define precisely the region of the C4 molecule which interacts with C5 in the C5 convertase. It is known that the difference does not lie within the most polymorphic section of the C4d region of C4A6 (Dodds *et al.*, 1986*b*). A knowledge of the portion of the C4 molecule involved in C5 convertase formation leads to the possibility of intervention at this step in complement activation. This could prove useful medically, as C5a is a major mediator of inflammation. Inhibition of C5a generation without inhibition of classical-pathway activation before this step would allow the clearance of immune complexes to proceed normally, but considerably reduce the inflammatory processes brought about by complement.

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